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Histological and Immunohistochemical Analyses of the Pulmonary Pathology That Occurs as a Direct Result of Acute Lethal Graft-Vs.-Host Disease

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HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES OF THE PULMONARY PATHOLOGY THAT OCCURS AS A DIRECT RESULT OF ACUTE LETHAL GRAFT-VS.-HOST DISEASE

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LIST OF ABBREVIATIONS

BAL	Bronchoalveolar Lavage
BMT	Bone Marrow Transplantation
BU	Busulphan
CMV	Cytomegalovirus
CsA	Cyclosporine A
CY	Cyclophosphamide
ELAM-1	Endothelial Leucocyte Adhesion Molecule
FACS	Flow Cytometric Analysis
GVHD	Graft-versus-Host Disease
GVHR	Graft-versus-Host Reaction
HLA	Human Leucocyte Antigen
ICAM-1	Intercelllular Adhesion Molecule
IFN-γ	Interferon-Gamma
IP	Interstitial Pneumonitis
LB	Lymphocytic Bronchiolitis/Bronchitis
LFA-1	Lymphocyte Function Related Antigen
LUT	Look-Up Table
MHC	Major Histocompatibility Complex
MTX	Methotrexate
TBI	Total Body Irradiation
TNF-α	Tumor Necrosis Factor - Alpha
VCAM-1	Vascular Cell Adhesion Molecule

VLA	Very Late Activation Antigen

GLOSSARY

ED1:	Macrophages, monocytes, and dendritic cell intracellular antigen
ED2:	Macrophage surface differentiation antigen
MOM/3F12/F2:	Granulocytes
MOPC 21:	IgG_1
OX6:	Ia antigen
OX8:	Cytotoxic T cells and NK cells
OX12:	Kappa chain
OX19/52:	Pan T cells
OX39:	Interleukin-2 receptor
OX41:	Alveolar and activated macrophages
OX42:	Pulmonary interstitial macrophages
UPC 10:	IgG_{2a}
W3/13:	T cells and NK cells
W3/25:	Helper T cells and macrophages

DEDICATION

To others who find themselves in a foreign land

endlessly, relentlessly swimming upstream

to accomplish the impossible -

isolated from others

with the same

visions -

others who are capable of achieving greatness

inspite of all the obstacles

imposed by themselves and others...

do not give in -

keep believing

listen to your inner voice

even when its lonely whisper

conflicts with those around you -

you can rise above it all...

you can do it

EPIGRAPH

Imagination is more important than knowledge
Albert Einstein

CHAPTER I

INTRODUCTION

Bone marrow transplantation (BMT) is a complex therapeutic regimen used to treat hematologic and oncologic disorders. Autologous, syngeneic and allogeneic bone marrow transplants are performed routinely, although allogeneic transplantation is most prevalent due to the current sophistication of major histocompatibility complex (MHC) matching and the unavailability of syngeneic donors. Graft-vs.-Host Disease (GVHD) is the most frequent complication of allogeneic BMT and results in high rates of morbidity as well as mortality. GVHD develops when immunocompetent donor lymphoid cells are transplanted into an immunocompromised host where alloreactive donor T cells initiate an immune response to disparate host major and multiple minor histocompatibility antigens. The knowledge that donor T cells initiate GVHD, has prompted many researchers and clinicians to deplete T cell populations from the donor inoculum. Although T cell depletion reduces the incidence of GVHD, it often abrogates engraftment, promotes leukemic relapse, and exacerbates the occurrence of infection. The induction of GVHD may produce a number of deleterious clinical complications, yet undefined advantageous components of the disease appear essential to the long-term success and increased application of allogeneic BMT.

This dissertation is focused on the acute form of GVHD that develops within 100 days post transplant and is characterized by the presence of complex clinical symptoms and high mortality rates. Although the development of well-defined skin, liver and G.I. tract histopathologies are considered diagnostic of acute GVHD, mortality is most often associated with pulmonary complications. GVHD-induced pulmonary complications have been considered to be a consequence of viral, fungal and bacterial infection resulting from the deterioration of

other organ systems and systemic immune suppression. Previous research has implicated interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis as prevalent components of acute pulmonary GVHD, but no direct evidence has shown a correlation between the onset of these histopathologies or determined whether their presence marks the development of a pulmonary syndrome that occurs as a direct result of acute GVHD. The characterization of acute GVHD-induced pulmonary histopathology has been difficult due to the compounding influence of chemotherapeutic agents, immunosuppressive drugs, irradiation and/or overt infection.

The prevalence and severity of pulmonary complications following allogeneic BMT combined with the inconclusive findings of previous studies have led to the fundamental question is the lung a target organ of acute GVHD. The hypothesis is that interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis occur as a direct result of acute GVHD. The primary objectives are to determine the time course, anatomical location, histological progression and phenotypic components of acute GVHD-induced pulmonary histopathology. The results of these objectives were produced using the adult nonirradiated (DA × LEW) F_1 hybrid rat in the absence of chemotherapeutic agents, immunosuppressive drugs, and overt infection. F_1 hybrid rats were injected with 1×10^6 DA parental lymphoid cells/gram body weight which produced 100% morbidity and mortality by day 21. Neither syngeneically ($F_1 \rightarrow F_1$) injected nor noninjected F_1 control animals contained any observable or measurable histopathology. Consequently, noninjected F_1 animals were used as controls throughout my studies. In addition, acute GVHD and control pulmonary tissues did not exhibit evidence of bacterial, fungal or viral contamination as determined by light microscopic analysis in conjunction with specific tissue and immunohistochemical staining. All GVHD-induced animals were killed by ether overdose on days 3, 7, 10, 14 and between 15 - 21 following injection.

Prior to the development of flow cytometric analysis (FACS), variations within cell populations of histologically and immunohistochemically analyzed tissues were reported in a qualitative manner. However, the development and availability of FACS analysis provided a way to produce precise quantitative measurements of phenotypic variability. Subsequently, FACS analysis remains a widely used method for assessing the immunopathological processes occurring within a variety of diseased tissues.

Chapter III details the results provided by FACS analysis of antibody-labeled bronchoalveolar lavage (BAL) and collagenase-digested mononuclear cell suspensions obtained throughout acute GVHD. A wide panel of mouse anti-rat monoclonal antibodies was used to label BAL and collagenase-digested cell suspensions in preparation for FACS analysis. Variations within lung weights and cell yields were also determined to assess evidence of pulmonary pathogenesis. Although lung weights averaged 2.7g throughout acute GVHD, collagenase-digested cell yields increased from 7.5×10^6 (controls) to 28.2×10^6 (day 14 GVHD) which corroborated the significant infiltration of mononuclear cells observed in subsequent histological studies. BAL cell yields ranged from 2.0 - 11.7 $\times 10^6$ (controls) to 2.2 - 51.1 $\times 10^6$ (day 14 GVHD). FACS analysis conducted using 10 monoclonal antibodies and two isotype controls provided reliable labeling, but no consistent trends could be extrapolated from the results produced by any two sets of animals. Constraints concerning the time required to ensure cell viability and equipment availability made it impossible to assay more than one control and one acute GVHD-induced animal at any given time. Overall, FACS analysis of BAL and collagenase-digested cell suspensions was incapable of producing results that clearly delineated the histopathology induced by acute GVHD. Recently, clinical studies comparing the efficacy of BAL to transbronchial biopsies from lungs undergoing GVHD or allograft rejection have corroborated my findings.

The development and availability of computer-assisted image analysis have made it possible to quantify the results obtained from histologic and immunohistochemical studies. Consequently, FACS analysis was abandoned in order to conduct histological and immunohistochemical analysis on pulmonary tissues. In Chapter IV, H&E-stained paraffin sections (4 μm) of whole lobe lung tissues were used to assess the time course, anatomical location, and histological progression of the pulmonary histopathology that occurs as a direct result of acute GVHD. This study revealed that interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis are integral components of acute pulmonary GVHD. The onset of interstitial pneumonitis (IP) was characterized by the coalescence of infiltrated perivascular and alveolar mononuclear cells after dissolution of the adventitial limiting plate. Lymphocytic bronchiolitis/bronchitis (LB) developed just prior to the dissemination of perivascular infiltrates. Furthermore, the sequential development of acute GVHD-induced IP and LB duplicated the histopathology of lung allograft rejection. This study defined the lung as an important target organ of acute GVHD.

The perivascular infiltrates of IP are the most prominent and easily quantified histological feature of acute GVHD. Therefore, Chapter V describes the phenotypic components of perivascular mononuclear infiltrates throughout acute GVHD. A streptavidin-biotin method of immunohistochemical analysis was employed using 10 mouse anti-rat monoclonal antibodies on frozen whole lobe lung sections (4 μm). The density of antibody labeling (volume density) was quantified using computer-assisted image analysis. Results determined that the process of perivascular infiltration was a biphasic response characterized first by the influx of $ED1^+$, $OX8^+$, and $W3/25^+$ populations by day 7, and then $OX41^+$, $W3/13^+$, and $OX19/52^+$ populations by day 14. $OX6^+$ mononuclear cells were present within the perivascular spaces of control and GVHD animals at all time points tested. OX12, OX39 and

MOM/3F12/F2 were not quantifiable within perivascular spaces at any time point tested.

This dissertation has determined that the lung is an important target organ of acute GVHD. Data have been produced to demonstrate that: (1) FACS analysis of BAL cell suspensions may be able to suggest an ongoing immune process, but in the absence of transbronchial biopsy this method is incapable of determining the onset or progression of acute pulmonary GVHD prior to clinical symptoms occurring from the significant deterioration of normal pulmonary structure, (2) IP and LB occur as a direct result of acute GVHD, (3) IP develops prior to LB and is produced by the coalescence of perivascular and alveolar mononuclear cell infiltrates, (4) acute GVHD-induced IP and LB duplicate the histopathology of lung allograft rejection, and (5) the process of perivascular infiltration is characterized by a biphasic influx of class II^+, T cell and macrophage populations.

CHAPTER II

REVIEW OF RELATED LITERATURE

Historical Overview

Bone Marrow Transplantation

In 1891, Brown-Sequard and d'Arsonaval (quoted by Quine 1896) orally administered bone marrow to patients with leukemia-induced anemia in the first recorded attempts to use marrow as a therapeutic measure. The oral administration of bone marrow proved unsuccessful and clinical attempts were curtailed until 1937 when Schretzenmayr prepared fresh intramuscular injections of autologous or allogeneic bone marrow to treat anemic patients with malaria or helminthic infections (Schretzenmayr 1937). However, by 1939 intramuscular injections were known to be as unsuccessful as oral administration. These findings led Rasjek (according to Migdalska 1958) to inject marrow directly within the intramedullary cavity as treatment for patients with lymphatic leukemia and pernicious anemia (Migdalska 1958). Although Rasjek's attempts were not reported to be successful, Morrison and Samwick effectively treated a patient with aplastic anemia by modifying Rasjek's intramedullary protocol to administer three infusions of bone marrow that had been aspirated from the patient's brother (Morrison and Samwick 1940). While the intramedullary protocol appeared promising, Osgood, Riddle and Mathews refined the procedure by intravenously injecting fresh bone marrow into patient's with aplastic anemia (Osgood, Riddle, Mathews 1939). Subsequently, the intravenous protocol for administration of bone marrow was accepted as the optimal method for undertaking hematopoietic reconstitution and currently remains standard practice for allogeneic bone marrow transplantation (BMT).

Irradiation

The ability to produce hematopoietic chimerism following lethal doses of total body irradiation (TBI) was discovered between 1945 - 1949 as a result of government research on the effects of atomic warfare, and became the foundation of modern bone marrow transplantation. In 1951, Lorenz et al. discovered that lethally irradiated mice and guinea pigs were protected from certain death by the parenteral administration of syngeneic marrow (Lorenz et al. 1951). Subsequent research determined that allogeneic and xenogeneic bone marrow cell injections were also therapeutically effective in prolonging survival from 20 - 30 days (Lorenz, Congdon and Uphoff 1952, Lorenz and Congdon 1954). By 1956, several other studies used a variety of genetic markers to demonstrate that the colonization of host marrow by donor cells provided a protective effect against lethal irradiation (Lindsley, Odel and Tausche 1955, Ford et al. 1956, Mitchison 1956, Nowell et al. 1956). As a result of host marrow reconstitution by donor cells, Ford et al. introduced the term "radiation chimera" to describe an animal that carries a foreign hematopoietic system as a result of lethal irradiation followed by the transplantation of hematopoietic cells from another animal (Ford et al. 1956). The most effective means of producing the chimeric state was acknowledged to be the intravenous administration of bone marrow cells following irradiation. The number of allogeneic cells required to produce optimal hematopoietic chimerism was reported to be 10 - 80 times the number of syngeneic cells required (van Bekkum and Vos 1957).

Chimeric studies demonstrating that hematopoietic reconstitution could be achieved in an irradiated host following the intravenous administration of bone marrow cells were instrumental to the incorporation of irradiation as a pre-transplant regimen in contemporary allogeneic BMT. In 1957, Thomas et al. were the first to describe transient engraftment in humans by intravenously infusing large amounts of marrow following irradiation and

chemotherapy (Thomas et al. 1957). By 1959, Mathé et al. attempted to rescue six human victims of an irradiation accident using allogeneic marrow, and in 1963 reported the first long-term survival of a 26-year-old leukemic patient that displayed evidence of complete engraftment (Mathé et al. 1959, 1963). This patient suffered from acute as well as chronic GVHD, but eventually died without any evidence of leukemia 20 months post transplant from varicella encephalitis (Mathé 1963). Although these early recorded efforts at allogeneic BMT following irradiation became the foundation for modern clinical protocols, attempts at engraftment were usually unsuccessful because they occurred prior to the concepts of histocompatibility and immunosuppression.

Major Histocompatibility Complex

Immunological identity of self versus foreign tissues is based on the expression of cell-surface proteins known as major histocompatibility complex (MHC) antigens. Disparate major and minor histocompatibility antigens expressed on cells or tissues transferred from one patient to another are normally recognized by the recipient, leading to rejection in a host-vs.-graft reaction. However, immunologically competent cells within the graft can also produce an immune response in the opposite direction, producing a Graft-vs.-Host Reaction (GVHR). MHC major and minor antigenic disparity between recipient and donor are the most important risk factors for the induction of graft failure and Graft-vs.-Host Disease (GVHD) (Reviewed in Ferrara and Deeg 1991).

Dausset discovered the human MHC, termed human leukocyte antigen (HLA) in the mid-1950's (Dausset 1958). Delineation of the MHC complex between the late 1960's and early 1970's, provided the knowledge required to immunologically select a bone marrow donor and facilitate the future success of allogeneic BMT. During the early 1970's, MHC class I

molecules, constitutively expressed by most tissues, became known as the major transplantation antigens. In contrast, MHC class II molecules were not recognized as the critical components of antigen recognition and alloreactive responses because contemporary serological assays had not been refined (Reviewed in Krensky et al. 1990). Consequently, it was not until 1974, when Zinkernagel and Doherty discovered that T cell recognition required both foreign antigen and the body's own MHC complex (Zinkernagel and Doherty, 1974). The de novo and upregulated expression of MHC class II antigens within host cutaneous, intestinal and hepatic compartments is considered to be a diagnostic component of acute GVHD (see Target Organs, p. 24). MHC compatibility between donor and host remains essential to the long-term survival of patients following allogeneic BMT, and the continued implementation of BMT as a therapeutic option.

Cytoreductive Therapy

Pre-transplant conditioning is used to: (1) promote graft survival by inducing immunosuppression through the elimination of host immunocompetent cells, (2) provide an anti-cancer effect, and (3) induce myeloablation to provide "room" for grafted cells. These goals are most often achieved by irradiation and the administration of chemotherapeutic agents (summarized up to 1963 by van Bekkum and de Vries 1967). In 1960, Beilby et al. provided the first evidence that chemotherapy could be used to perform allogeneic transplants (Beilby et al. 1960). A patient with Hodgkin's disease developed severe marrow hypoplasia after receiving aminochlorambucil, but an infusion of bone marrow from the patient's sister resulted in a 24% increase of donor erythrocytes 6 months post transplant.

By 1970, a series of animal and clinical studies developed a rationale for using cyclophosphamide (CY) as a preparative regimen for human allogeneic BMT (Santos, Owens

and Sensenbrenner 1964; Santos et al. 1970). Further murine and rat studies demonstrated the combined efficacy of busulphan (BU) and CY during human allogeneic BMT (Santos 1966a, b; Santos and Hagshenass 1968; Sensenbrenner et al. 1968; Santos and Tutschka 1974a, b; Tutschka and Santos 1975a, b; Tutschka and Santos 1977). Cyclophosphamide appeared to be an excellent immunosuppressive agent with anti-tumor properties, but did not appear capable of removing enough host cells to make "room" for the graft. In contrast, BU was lacking immunosuppressive capabilities but had excellent anti-tumor properties and appeared to remove enough cells to provide "room" for engraftment. The concept of "room" referred to the physical space required for the accommodation of transplanted cells and the non-immunological factors necessary to promote engraftment (Santos 1974). The use of cytoreductive chemotherapeutic drugs, such as BU and CY, remains a crucial part of the allogeneic BMT protocol.

Graft-vs.-Host Reaction, Secondary Disease, Runt Disease

The Graft-vs.-Host reaction was first described by James B. Murphy in 1916, fifteen years after Brown-Sequard and d'Arsonaval attempted to use bone marrow to cure leukemia-induced anemia (Murphy 1916, Quine 1896). Murphy inoculated the chorioallantoic membranes of 7-day-old chicken embryos with fragments of spleen and bone marrow obtained from adult chicken donors. The inoculation induced a marked host splenomegaly as well as the development of varying sized whitish nodules on spleen and chorioallantoic membranes. Murphy attributed this immunologic process to stimulation of the host's spleen and the subsequent proliferation of host leukocytic elements in additional tissue sites (Murphy 1916). Murphy's preliminary observations were confirmed and extended by Danchakoff through an extensive histological analysis of the effects produced by grafting adult spleen cells to chorioallantoic membrane (Danchakoff 1918, 1920). She observed that the capacity to stimulate host mesenchymal tissues was confined to spleen, liver and marrow cells obtained

from a donor of the same species. Danchakoff favored the belief that proliferation of the host's spleen was induced by the blood-borne products of metabolism produced from viable transplanted cells. The observations by Murphy and Danchakoff formulated the definition of GVHR.

In 1955, Barnes and Loutit were first to report the development of an immunologic syndrome with lethal consequences that appeared unrelated to the primary cause of disease, termed "secondary disease" (Barnes and Loutit 1955). Lethally irradiated CBA mice given syngeneic spleen cells survived radiation injury. However, 9 out of 16 lethally irradiated CBA mice injected with A strain spleen cells died between 30 - 100 days post transplantation. Although the mice were recovering from radiation injury and marrow aplasia, an injection of allogeneic marrow induced mortality from a secondary disease that was characterized by severe diarrhea, weight loss, skin lesions and liver abnormalities (Barnes and Loutit 1955, Cohen, Vos and van Bekkum 1957). Trentin also demonstrated that mice given syngeneic bone marrow after TBI were provided with long-lasting protection and a minimum of complications (Trentin 1956). While the injection of allogeneic marrow into lethally irradiated mice resulted in weight loss, diarrhea, skin lesions and significant mortality between 20 - 100 days. Subsequently, the induction of this "secondary disease" syndrome was confirmed in rats, dogs, primates and man (studies compiled by van Bekkum and de Vries 1967). The severity and accelerated development of secondary disease was induced by increasing the number of lymphoid cells injected with bone marrow (Santos and Cole 1958). These observations provided evidence to support future acknowledgements of a GVHD mechanism.

In 1956, Billingham and Brent described a similar "secondary disease" as "runt disease" after conducting studies on the induction of tolerance using murine skin homografts

(Billingham and Brent 1957,1959). Newborn host CBA mice were intravenously injected with a spleen cell suspension obtained from an A strain murine donor. Mortality was low in the neonatally inoculated hosts and most recipients developed into normal adults tolerant of A skin. Yet, post-mortem examination revealed hypoplasia in most lymphoid organs. In other murine strain combinations, recipient mice stopped growing, became emaciated and frequently suffered from diarrhea. Although most of the affected mice died within a few days, some survived for a month or two in a chronically ill state. These mice were characterized by retarded growth and development, sparse abnormal-looking fur, varying degrees of lymphatic hypoplasia, erythematous desquamating dermatitis that produced a mincing gait, and focal necrosis of hepatic cells (Billingham and Brent 1957, 1959).

Three specific lines of evidence suggested that the "runt disease" described by Billingham and Brent resulted from a graft-vs.-host reaction: (1) the severity of the disease was directly proportional to the genetic disparity between donor and host strains, (2) the severity of the disease was heightened and accelerated if the spleen cells used in the donor innoculum were previously sensitized against the antigens of the intended recipient, and (3) the disease did not develop if spleen cells from an F_1 hybrid donor were injected into parental strain newborn mice (Billingham and Brent 1957,1959). Both secondary disease and runt disease appeared to be the result of immunologically competent cells within the donor innoculum reacting against foreign transplantation (MHC) antigens of the immunologically immature or immunocompromised host (Billingham and Brent 1957, 1959). Subsequent studies confirmed that secondary disease was immunologically equivalent to runt disease and both were eventually shown to be GVHD.

Graft-vs.-Host Disease

The most life-threatening complication of allogeneic BMT is Graft-vs.-Host Disease (GVHD), which occurs in more than 80% of unrelated HLA-matched transplants with a 50% mortality rate. By 1966, several reports documenting the development of GVHD led Billingham to formulate the requirements for induction of GVHD: (1) the graft must contain immunologically competent cells, (2) the host must express tissue antigens that are absent in the graft donor, appear foreign to the donor, and are capable of antigenic stimulation, and (3) the host must be incapable of mounting an effective immunologic response against the donor graft (Billingham 1966-67).

By the early 1960's, Medawar had postulated that small lymphocytes were the immunologically competent donor cells necessary to induce GVHD, and Gowans was the first to prove this hypothesis (Medawar 1963, Gowans 1962). Gowans demonstrated that a suspension of thoracic duct lymphocytes, depleted of large lymphocytes by incubation overnight at 37^0 C, retained GVHD reactivity when injected into adult F_1 hybrid rats (Gowans 1962). McGregor expounded on Gowan's data by showing that bone marrow could generate the immunologically competent small lymphocytes capable of initiating a lethal graft-vs.-host reaction (McGregor 1968). In addition, McGregor was able to show that although bone marrow cells were incapable of directly producing GVHD, some bone marrow stem cells can become GVHD-inducing T cells after thymic influence. Subsequently, Owens and Santos provided quantitative data to show that various tissues displayed a greater ability to produce lethal GVHD in the cyclophosphamide mouse than others: peripheral blood > lymph node > spleen > marrow (Owens and Santos 1968). Cells isolated from the thymus were relatively inactive. These data extended and confirmed the earlier observations of Billingham and Silvers (Billingham and Silvers 1962).

A consistent manifestation of GVHD in all tested species was shown to be splenomegaly, which peaked 6 - 10 days after the injection of immunocompetent donor cells, and provided a quantitative assay to assess the severity of GVHD (Simonsen 1962). In 1962, Simonsen developed the spleen index assay to determine the degree of splenomegaly. Absolute spleen weights of recipient animals were compared to uninjected littermate controls, and relative spleen weights were determined by comparing spleen/body weight ratios. Spleen index was calculated for each recipient animal by dividing its relative spleen weight by the relative spleen weight for control littermates. An index of 1.0 indicated no change in spleen weight, whereas an index of 2.0 determined a doubling of spleen weight. The spleen index assay remains the most commonly used parameter to assess the development of GVHD in experimental models.

The most severe form of acute GVHD occurs when genetic disparity exists between donor and host, and a large number of mature T lymphoid cells are included in the donor inoculum. Evidence that GVHD is directly proportional to the presence of donor Thy 1^+ T cells was demonstrated by pretreating parental strain lymphoid cells with anti-Thy 1^+ antibody plus complement in H-2 different neonatal F_1 mice or adult irradiated F_1 mice (Cantor 1972, Sprent, von Boehmer and Nabholz 1975). Subsequently, many laboratories demonstrated that GVHD produced across MHC disparity was caused by mature T cells (Tyan 1973, Trentin and Judd 1973, Rodt, Thierfelder and Eulitz 1974, Muller-Ruchholtz, Wottge and Muller-Hermelink 1976, Onoe, Fernandes and Good 1980, Vallera et al. 1981).

Additional studies have indicated that lethal GVHD in irradiated adult mice can be elicited by multiple minor histocompatibility antigen differences as well as full MHC disparity (Korngold and Sprent 1978, Halle-Pannenko et al. 1978, Hamilton, Bevan and Parkman 1981,

O'kunewick et al. 1982). Korngold and Sprent treated marrow cells with anti-Thy 1.2 antibody and complement to deplete mature T cell populations while leaving pre-T cells intact. The transplantation of T-cell-depleted B10.BR marrow into allogeneic irradiated recipients across only minor histocompatibility differences failed to cause GVHD. Consequently, this study demonstrated that GVHD to minor antigens is produced by mature T cells contaminating donor marrow (Korngold and Sprent 1978).

The subset of mature T cell required to produce GVHD was determined by adding separate T-cell populations to the donor bone marrow. The first study to determine the role of T cell subsets in producing lethal GVHD across a full MHC barrier was conducted in rats (Mason 1981). Depletion of $OX8^+$ ($Lyt\text{-}2^+$) (cytotoxic T) donor cells produced a population of $W3/25^+$ (helper T) cells that were very potent inducers of lethal GVHD. Although the transfer of purified $OX8^+$ cells produced a weaker but eventually lethal GVHD.

In a murine model, highly purified $L3T4^+$ and $Lyt\text{-}2^+$ T cell populations were produced using antibody combined with complement depletion and positive panning on antibody-coated plates. These purified populations indicated that both subsets were active during the induction of GVHD (Sprent and Schaefer 1985). Purified $Lyt\text{-}2^+$ T cells were fully capable of producing GVHD in murine models of allelic or mutant MHC class I differences (Korngold and Sprent 1985, Sprent et al. 1986). Additional studies using different strain combinations showed that $Ly\text{-}2^+$ cells consistently produced GVHD to minor histocompatibility antigens, while $L3T4^+$ were able to induce GVHD in only a minority of the same strain combinations. Consequently, $Ly\text{-}2^+$ T cells were characterized as the only mediators of anti-class I GVHD, and $L3T4^+$ T cells were characterized as the initiators of anti-class II GVHD. Both populations were capable of inducing GVHD in murine strain combinations with full H-2

genetic disparity (Korngold and Sprent 1983, 1987).

Further murine and human studies using MHC-matched marrow demonstrated that cytotoxic T cells directed against minor histocompatibility antigens were the initiators of GVHD. The identification of disparate minor histocompatibility antigens required MHC (H-2) priming and were shown to be MHC-restricted (Bevan 1975, Hamilton et al. 1981). Cytotoxic T cells directed at minor histocompatibility antigens were also detected in patients with acute GVHD following BMT (Goulmy et al. 1984). These T cells were cloned and used as typing reagents to identify 4 minor histocompatibility antigens. Data compiled from rat, murine and human studies have established that specific T cell subsets initiate the development of acute GVHD in response to disparate major and/or minor histocompatibility antigens.

Immunosuppression

Immunosuppression is required to reduce the occurrence and severity of acute GVHD in all patients undergoing allogeneic BMT. Common methods of immunosuppression include the use of cyclosporine A (CsA) and methotrexate (MTX), as well as T cell depletion of the donor inoculum. Since donor T lymphocytes are acknowledged to initiate GVHD, immunosuppression is designed to block the activation of T cells or eliminate them.

Cyclosporine A. Cyclosporine A, a fungal metabolite, was introduced as an immunosuppressive drug in the 1970's. Initial reports suggested that established acute GVHD was lessened after the administration of CsA, but additional studies demonstrated that symptomatic acute GVHD did not respond to CsA administration (Powles et al. 1980, Tutschka et al. 1983). In contrast, CsA prevented the development of acute GVHD after allogeneic BMT in rats prepared with TBI or CY, and acute GVHD did not appear when the drug was

discontinued (Tutschka et al. 1979). In addition, acute GVHD may appear after the withdrawal of immunosuppressive drugs, such as CsA (Powles et al. 1980).

The mechanism of CsA is still not completely understood, but the drug appears to inhibit T cell activation. CsA has inhibited the proliferative response to: (1) concanavalin A in murine, rat and human studies (Leoni, Garcia and Allison 1978, Burckhardt and Guggenheim 1979, White et al. 1979, Larsson 1980), (2) to phytohemagglutinin in porcine, rat and human models (Leoni, Garcia and Allison 1978, Burckhardt and Guggenheim 1979, White et al. 1979,) and (3) pokeweed mitogen in humans (Leoni, Garcia and Allison 1978). Several studies have indicated that CsA inhibits the synthesis of interleukin-2, interleukin-2r, interleukin-3, interleukin-4, interferon-gamma, tumor necrosis factor-alpha, and potentially interleukin-1 mRNA at the transcriptional level (Elliot et al. 1984, Granelli-Piperno, Inaba and Steinman 1984, Granelli-Piperno, Andrus and Steinman 1986, Granelli-Piperno, Keane and Steinman 1988, Goldfeld et al. 1993). In addition to remarkable immunosuppressive effects, CsA appeared to promote a faster rate of engraftment and decreased the occurrence of mucositis (Atkinson et al 1983). Although the use of CsA is limited by the induction of severe nephrotoxicity (Barrett et al. 1982).

Methotrexate. Methotrexate is a folic acid antagonist with immunosuppressive and anti-neoplastic activity that may prevents the division and clonal expansion of activated T cells by deactivating activated macrophages or affecting the function of adenosine receptors. In 1970, Storb observed that the administration of MTX to irradiated dogs following syngeneic marrow 90% induced survival without the production of acute GVHD (Storb et al. 1970). In contrast, dogs that did not receive MTX post-transplant developed a 55% incidence of acute GVHD and underwent a 45% decrease in survival rate. As a result of these studies, clinical.

trails were conducted to determine the efficacy of MTX administration following BMT (Sullivan et al. 1986). Patients that did not receive MTX developed hyperacute GVHD with exacerbated infectious complications and poor graft function.

In 1983, Deeg et al. reported that there was no statistical difference between the immunosuppressive capabilities of CsA and MTX as compared to the development of GVHD or prolonged survival (Deeg et al. 1983). However, the survival rate in dogs following allogeneic BMT was increased from 6% with either CsA or MTX to 35% when the drugs were combined (Deeg et al. 1982). Subsequently, Storb et al. conducted a prospective randomized study in 48 patients with severe aplastic anemia to compare the single or combined use of CsA and MTX (Storb et al. 1986 a). Results showed that a combination of CsA/MTX significantly reduced the incidence of severe acute GVHD to 18% as compared to 53% for CsA or MTX alone. Similar results were obtained in patients with acute lymphoblastic leukemia, acute nonlymphoblastic leukemia, chronic myelocytic leukemia, or chronic granulocytic leukemia (Storb et al. 1986 b, 1988, 1989). Consequently, the combination of CsA and short-course MTX is considered standard protocol for acute GVHD prophylaxis following allogeneic BMT.

T cell depletion. In addition to post- transplant immunosuppression, the pre-transplant depletion of T cells from the donor inoculum has also been shown to prevent the development of acute GVHD. This concept worked well in murine studies conducted by Korngold and Sprent, and as a result several clinical transplantation centers incorporated a variety of techniques to eliminate T cells throughout the 1980's (Korngold and Sprent 1978). The techniques developed for T cell depletion included using anti-T-cell antibodies alone, in combination with heterologous or homologous complement, conjugated to immunotoxins, or combined with immunomagnetic beads (Vallera et al. 1981, Prentice et al. 1984, Martin et al.

1985, Apperley et al. 1986, Mitsuyasu et al. 1986, Atkinson et al. 1987, Filipovich et al. 1987, Vartdal et al. 1987). In addition, counterflow centrifugal elutriation, density fractionation, and differential agglutination with soybean lectin followed by rosetting with sheep erythrocytes were all T cell depleting methods that did not rely on the use of antibodies (Reisner et al 1981, O'Reilly et al. 1985, DeWitte et al. 1986, Lowenberg et al. 1986, Wagner et al. 1988).

The data compiled from clinical centers confirmed that the occurrence and severity of GVHD were significantly reduced using T-cell depleted allogeneic bone marrow transplants. However, these T-cell depleted transplants produced an increase in graft failure as well as leukemic relapse, and delayed immune reconstitution (Prentice et al. 1982, Filipovich et al. 1984, Martin et al. 1985, 1988, O'Reilly et al. 1985, Trigg et al. 1985, Henslee et al. 1987, Maraninchi et al. 1987). The primary disadvantage of T-cell depleted marrow transplants in patients preconditioned with TBI was the severe prolonged immunoincompetence that resulted in a heightened susceptibility to opportunistic infection (Weiner and Dicke 1987). Eventually, it became apparent that the development of acute GVHD had a positive impact on the rate of engraftment and the elimination of malignant cells. These findings have compounded the necessity for defining the complex immunologic phenomenon of acute GVHD.

Historical Summary

The number of allogeneic BMT's performed worldwide increased exponentially during the 1980's. In 1990 alone, over 4000 allogeneic transplants were estimated to have been completed. The standard contemporary protocol for conducting allogeneic BMT, requires the use of supralethal chemotherapeutic drugs and radiation in an attempt to "cure" the tumor-bearing host, which is followed by "immunologic rescue" from clinically-induced hematopoietic failure through the adoptive transfer of marrow stem cells from a healthy MHC-matched donor.

Bone marrow transplantation is now considered to be an accepted therapeutic option for patients with chronic myelogenous leukemia, aplastic anemia, severe combined immunodeficiency and oncologic disorders that are resistant to previous therapies. Patients under 40 who have an HLA-identical sibling donor and are transplanted for chronic myelogenous leukemia while in the chronic phase of disease, or those with acute lymphoblastic leukemia in remission are the most likely to enjoy disease-free survival. The establishment of an International Bone Marrow-Donor Registry has increased the availability of HLA-matched marrow from unrelated donors. However, the increased use of MHC-matched unrelated allogeneic BMT has focused attention on the immunobiologic complexities and consequences of GVHD, which remains the primary immunologic complication and leading cause of death following allogeneic BMT.

Acute Graft-versus-Host Disease

Experimental Models

Murine vs. rat. The use of animal models has been critical to defining and understanding the pathogenesis as well as pathogenetics of GVHD. Although canine, porcine, rabbit, hamster, guinea pig, monkey, murine, rat and human models have been used to study the immunopathogenesis of GVHD, murine and rat models are the most extensively used. Proponents of the murine model rely on the availability of numerous inbred strains that allow for control over genetic variables. In addition, there is a wide variety of cellular and molecular probes readily available. In contrast, proponents of the rat model believe that the rat's intermediate size provides technical advantages over the murine model (Gill et al. 1989). Although there are fewer inbred strains available, the rat can provide large amounts of cells as well as serum, and relatively large internal organs can easily be assessed for histological and immunohistochemical studies.

Lewis/BN and Lewis/DA rat strain combinations are considered to be optimal rodent models for GVHD experimentation. In 1966, Elkins and Palm demonstrated that only rats with antigenic variability at the *Ag*-B locus of the major histocompatibility complex can elicit graft-versus-host reactions using spleen cells from unsensitized donors (Elkins and Palm 1966). Lewis, BN and DA rat strains all contain variant alleles of the *Ag*-B locus, and have at least one locus closely linked to the *Ag*-B site that codes for minor alloantigens (Marshak et al. 1977). In 1975, Atkins and Ford estimated the proportion of rat lymphocytes responsive to *Ag*-B antigens in the GVH reaction (Atkins and Ford 1975). Radioactively labeled parental and F_1 thoracic duct lymphocytes were intravenously injected into F_1 rats. The amount of labeled cells retained within recipient spleens allowed Atkins and Ford to calculate that approximately 12% of parental lymphocytes reacted to the products of one *Ag*-B haplotype. It was also estimated that 4.5-6.0% of donor T cells were specifically responsive to the recipient's *Ag*-B antigens. Additional studies using anti-idiotypic serum in Lewis and DA rats confirmed major and minor histocompatibility antigen disparity at the *Ag*-B locus (Binz and Wigzell 1975). Consequently, the Lewis/DA strain combination was used throughout this dissertation to investigate the immunohistopathology of acute GVHD.

Irradiated vs. parental $\rightarrow F_1$ hybrid. The two most common experimental models used to induce GVHD incorporate total body irradiation and the intravenous injection of parental lymphoid cells into F_1 ($P \rightarrow F_1$) mice or rats. The irradiated protocol produces the classic radiation chimera by destroying the recipient's marrow stem cells and immune system prior to the intravenous injection of donor lymphoid cells. In the radiation chimera, weight loss and mortality are the primary assays used to determine the onset and severity of GVHD. Although, the development of well-defined histopathologies within lymphoid and nonlymphoid organs are also used to assess the severity of GVHD (Rappaport et al. 1979). The irradiated model is

used extensively because it is believed to duplicate the clinical setting.

In the P $\rightarrow$ F_1 model, the F_1 host is tolerant of parental donor antigens and can not reject the injected donor cells. In contrast, the donor cells are stimulated by alloantigens expressed on F_1 host cells which induces GVHD. The injection of more than 100 $\times$ 10^6 donor lymphoid cells into F_1 animals produces a GVHD that strongly resembles the disease induced in irradiated animals. The injection of fewer than 20 - 60 $\times$ 10^6 donor lymphoid cells into F_1 animals induces a weak GVHD that does not produce wasting or skin lesions. In the (P$\rightarrow$ F_1) model, the primary assays used to determine the onset and severity of GVHD are: (1) spleen index to determine the degree of splenomegaly (Simonsen 1962), (2) developing histopathology within GVHD target organs (see Target Organs p. 24), and (3) immunosuppression (Shearer and Pollisson 1980). Spleen index is used and reflects the donor-cell induced recruitment of host cells (Howard, Michie and Simonsen 1961, Fox 1962, Elie and Lapp 1977). Immunosuppressive assays are used often because the F_1 immune system is intact prior to the injection of parental lymphocytes. The P$\rightarrow$ F_1 model makes it possible to study ongoing immunopathogenesis in direct relationship to the development of acute GVHD.

In either the irradiated or P$\rightarrow$ F_1 modes, it is disparity between major and minor histocompatibility antigens that determines the severity of graft-versus-host immune dysfunction (Rolink, Pals and Gleichmann 1983, Shearer and Levy 1983, Moser, Sharrow and Shearer 1988, Moser et al. 1987). Acute GVHD produced across disparate class I and II MHC antigens is characterized by the severe immune deficiency of T- and B-cell functions, the direct attack of donor cells on lymphohematopoietic tissues, and a reconstitution of the lymphohematopoietic system with donor-derived cells (van Eleven et al. 1981, Rolink et al. 1982, Via, Sharrow and Shearer 1987, Knobloch and Dennert 1988, Moser, Sharrow and Shearer 1988). Class II

disparity is characterized by the limited engraftment of donor cells, limited T-cell functional deficiency, polyclonal B-cell activation and production of autoantibodies (van Eleven et al. 1981, Rolink et al. 1982, van Rappard-van der Veen, Rolink and Gleichmann 1982, Rolink, Pals and Gleichmann 1983, Via, Sharrow and Shearer 1987, Knobloch and Dennert 1988). Neither class I nor minor histocompatibility disparity can produce an acute GVHD in the $P \rightarrow F_1$ model (Rolink, Pals and Gleichmann 1983, Moser, Sharrow and Shearer 1988, Shearer and Levy 1983).

Onset and Clinical Grading of GVHD

In both animal and human models, GVHD exists in acute and chronic forms. Acute GVHD usually develops within the first 100 days post transplantation and often precedes the onset of chronic GVHD in the following ways: (1) acute phase disease is not followed by the chronic form, (2) acute disease may gradually progress into the chronic phase (progressive GVHD), (3) acute disease may appear in remission for a length of time followed by the sudden onset of chronic disease (quiescent GVHD), or (4) chronic GVHD may appear in the absence of acute phase disease (de novo GVHD) (Sullivan 1981). The onset of GVHD is usually preceded by signs of engraftment, but occasionally GVHD may appear in the complete absence of engraftment (empty marrow, low peripheral blood cell counts). Consequently, clinical and histological characteristics are more definitive parameters of hematopoietic reconstitution than the onset of acute GVHD.

In 1974, Glucksberg et al. established a clinical grading system to assess the development of acute GVHD based on pathological and clinical parameters (Glucksberg et al. 1974). This grading system remains instrumental to the clinical management of allogeneic BMT patients, and classifies the severity of clinical symptoms from grades I - IV. Grade I consists of only skin pathology. The onset of extensive skin pathology, liver or intestinal tract symptoms,

and impairment of clinical performance, either alone or in any combination, advances the grade of acute GVHD from II - IV. Overall, the grade of acute GVHD profoundly affects a patient's prognosis following allogeneic BMT.

Target Organs

Cutaneous GVHD. Skin, intestinal tract, and liver are the main target organs of acute GVHD. Cutaneous symptoms signal the onset of acute GVHD, and include itching, pain upon pressure, and retroauricular as well as palmoplantar erythema. More is known about the cutaneous histopathology of acute GVHD than any other target organ pathology because frequent biopsies can be taken to assess temporal changes throughout the disease without producing serious morbidity.

The clinical and histological grading of acute cutaneous GVHD have been classified as: (1) Grade I - Macularpapular rash on less than 25% of total body surfaces with basal cell vacuolization, (2) Grade II - Macularpapular rash on more than 25% of total body surfaces with basal cell vacuolization, and single necrotic keratinocytes that appear as mummified cells adjacent to satellite lymphocytes, (3) Grade III - Erythroderma with subepidermal clefts and numerous keratinocytes, and (4) Grade IV - Toxic epidermal necrolysis with necrosis of the entire epidermis and complete desquamation (Glucksberg et al. 1974). Consequently, epithelial necrosis is considered to be diagnostic of acute GVHD (Sale et al. 1977). In spite of the well-defined grading system, signs of acute cutaneous GVHD are difficult to distinguish from viral infections, and cytoreductive drugs, immunosuppressive therapy, allergic antibiotic reactions, or radiation damage (Sale et al. 1977).

Throughout the 1980's, several studies observed that class II MHC antigens,

normally found only on dendritic Langerhans cells, were expressed on keratinocytes throughout acute GVHD (Breathnach and Katz 1983, Volc-Platzer et al. 1988). In 1982, Lampert et al. were among the first to assess the expression of class II MHC antigens and determine the phenotype of lymphoid cells present within epidermal infiltrates (Lampert et al. 1982). Immunohistochemical analysis of skin biopsies taken from acute GVHD patients provided evidence that class II expression was observed on keratinocytes and dermal macrophages, while the population of dendritic Langerhans cells was significantly reduced. $OKT8^+$ (cytotoxic T) cells were the predominant phenotype within lymphoid infiltrates. These observations were later confirmed by Lever et al. during immunohistochemical studies on skin biopsies taken from acute GVHD patients (Lever et al. 1986). Furthermore, the class II antigen expressed on keratinocytes was shown to be synthesized by the keratinocytes themselves and not derived from donor lymphocytes or absorbed from host Langerhans cells (Breathnach and Katz 1983).

By 1986, several studies attempted to define the role of T cells observed within cutaneous lesions and establish their proximity to class II^+ keratinocytes. The expression of class II on keratinocytes appeared directly related to the presence of lymphocytic infiltrates containing activated T cells (Auböck et al. 1986). Both $Ly\text{-}2^+$ and $L3T4^+$ T cells were shown to be capable of initiating epidermal cell necrosis, while predominately $L3T4^+$ produced the lichenoid hyperplastic reaction common to acute GVHD (Piguet et al. 1987a).

In 1988, studies documented that interferon-gamma (IFN-γ) produced by activated donor T cells induced the de novo expression of class II on human epidermal keratinocytes (Dustin et al. 1988, Niederwieser et al. 1988). Subsequent immunohistochemical staining of human biopsies confirmed that IFN-γ also upregulated the expression of intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leucocyte

adhesion molecule-1 (ELAM-1) on epidermal keratinocytes (Dustin et al. 1988, Norton et al. 1991, Norton and Sloane 1991). In addition to upregulating class II and adhesion molecule expression, IFN-γ also upregulated tumor necrosis factor- alpha (TNF-α) production by local macrophages (Collart et al. 1986). Piguet et al. have demonstrated that mice undergoing acute GVHD did not develop cutaneous lesions and had significantly reduced mortality rates after being treated with recombinant murine anti-TNF-α (Piguet et al. 1987b).

Intestinal GVHD. Diarrhea is a characteristic sign of acute GVHD in experimental animals and humans. In moderate GVHD, diarrhea is absent unless food is ingested, and anorexia becomes a prominent symptom. The most severe form of acute GVHD produces profuse watery diarrhea, protein loss, intestinal bleeding, mucosal denudation, edema of the intestinal wall, increasing pain and sepsis. Diarrheal fluid contains high concentrations of protein, cellular debris, and occult blood, resulting in a decrease of serum protein as diarrhea persists (Weisdorf et al. 1983). Acute GVHD most often affects the ileum and cecum, and viral infections may produce radiologic findings that mimic those produced by acute GVHD (Epstein et al. 1980, Snover et al. 1985, Jones et al. 1988).

The clinical grading of intestinal GVHD is defined as: (1) Grade I - no gut involvement, (2) Grade II - diarrhea 500-1000 mL/day and a mild decrease in clinical performance, (3) Grade III - diarrhea 1000-1500 mL/day and a marked decrease in clinical performance, and (4) Grade IV - diarrhea exceeding 1500 mL/day and an extreme decrease in clinical performance (Glucksberg 1974). In experimental animals early pathologic features include increased crypt cell mitotic activity, crypt lengthening and increased counts of intraepithelial lymphocytes as well as mucosal mast cells (Wall, Rosenberg and Reilly 1971, Elson, Reilly and Rosenberg 1977). Late pathological features include villus atrophy, increased

crypt cell turnover, crypt cell necrosis and the loss of mucosal lymphoid cells (Elson, Reilly and Rosenberg 1977, Mowat and Ferguson 1982, Snover et al. 1985).

Murine and rat studies have also determined that an upregulation of class II MHC antigens by enterocytes is another characteristic of acute GVHD (Mason, Dallman and Barclay 1981, Barclay and Mason 1982, Guy-Grand and Vassalli 1986). In control animals, class II MHC antigens were constitutively expressed at low levels on enterocytes although class II expression was absent on normal crypt cells. However, the development of acute GVHD upregulated class II expression on villus enterocytes and induced the de novo expression of class II on crypt cells (Barclay and Mason 1982, Dilly and Sloane 1987).

Three (C3H × DBA/2) F_1 murine models of acute GVHD were used to determine if T cell subsets induced enteropathy and upregulated class II MHC antigens throughout the development of acute intestinal GVHD (Guy-Grand and Vassalli 1986). $CD4^+$ T cells induced crypt hyperplasia and class II expression in irradiated recipients, while $CD8^+$ T cells induced histopathology in unirradiated adult and neonatal recipients. $CD8^+$ T cells infiltrated the mucosa more efficiently, while $CD4^+$ T cells were markedly increased within intraepithelial lymphocyte populations and induced hyperplasia of mucosal mast cells. Immunohistochemical studies of human rectal GVHD tissues following allogeneic BMT determined that the number of $CD8^+$ T cells increased in both lamina propria as well as epithelium, and class II MHC antigen expression increased on epithelial cells in 7 out of 8 GVHD patients (Dilly and Sloane 1987). In contrast to the increased number of $CD8^+$ T cells within human rectal tissues, the number of $CD4^+$ T cells remained the same.

The induction of class II MHC antigens on villus enterocytes and crypt cells,

appeared to be determined by the presence of activated donor T cells and the production of IFN-γ (Mowat 1989). Although the number of intraepithelial lymphocytes was increased in (CBA × BALB/c) F_1 mice with acute GVHD, the administration of anti-IFN-γ reduced crypt hyperplasia. These results suggested that IFN-γ production was required to produce GVHD-induced enteropathy. In addition to the corresponding effects of IFN-γ in cutaneous and intestinal GVHD, TNF-α has also been implicated as an effector mechanism of GVHD-induced enteropathy (Piguet et al. 1987b). None of the GVHD-induced (B10 × CBA) F_1 mice injected with recombinant murine anti-TNF-α developed enteropathic lesions characteristic of acute GVHD.

Adhesion molecules also appear to play important roles in the induction of acute GVHD enteropathy. Rectal biopsies from GVHD patients showed de novo ICAM-1 staining on the luminal surface of glandular epithelium (Norton et al. 1992). Although this staining was sometimes focal and variable in intensity it was present in all GVHD biopsies. In contrast, ELAM-1 staining was observed only on vascular endothelial cells within the most affected areas of the mucosa. Similar to ELAM-1, VCAM-1 staining was significantly positive on mucosal vascular endothelium and staining increased in proportion to the severity of GVHD. The de novo and upregulated expression of class II MHC antigens, the presence of IFN-γ and TNF-α induced histopathologies, the upregulation of adhesion molecules, and the extent of epithelial damage within cutaneous as well as intestinal organ compartments has suggested a common immunopathological pattern to acute GVHD.

Hepatic GVHD. Signs of cutaneous and intestinal GVHD precede the onset of hepatic involvement. Although over 80% of all patients develop hepatic dysfunction following allogeneic BMT, only half of hepatic disease is a direct result of acute GVHD (Reviewed by

McDonald et al. 1987). Chemotherapy, viral infections, drug-induced liver injury, bacteremia, and hypotension are all significant sources of hepatic damage post-transplant. GVHD-induced hepatic histopathology includes cholectasis, scattered hepatocellular necrosis and jaundice. Serum levels of alkaline phosphatase and total bilirubin are increased 20 times over normal limits, although serum hepatocellular enzymes are less elevated.

Clinical grading of hepatic GVHD is classified as: (1) Grade I - no liver involvement, (2) Grade II - bilirubin levels of 2 - 3 with a mild decrease in clinical performance, (3) Grade III - bilirubin levels of 3 - 15 with a marked decrease in clinical performance, and (4) Grade IV - bilirubin levels greater than 15 with an extreme decrease in clinical performance (Glucksberg 1974). Several studies have documented the progression of acute GVHD-induced liver histopathology. Clinical findings included mild nonspecific lobular hepatitis similar to viral or drug-induced hepatitis, and bile duct lesions were observed by light microscopy after 1-2 weeks of GVHD (Snover et al. 1984, Shulman et al. 1988). Bile duct lesions affected small septal as well as interlobular bile ducts, and were characterized by segmental destruction of the duct wall, irregular nuclear enlargement, and cytoplasmic swelling. As GVHD progressed, cholestatic changes of the acinus became more obvious and endothelial cells were destroyed.

Immunohistochemical studies have also been conducted to define the role of T cells, class II MHC antigen expression, and the presence of adhesion molecules throughout hepatic GVHD. Immediately post transplant the numbers of $T8^+$ and $T4^+$ cells were markedly reduced in portal areas. However, the development of acute GVHD caused an increase in $T8^+$ cells within portal tracts that did not exceed normal values, but were greater than post-transplant values (Dilly and Sloane 1985). Additional immunohistochemical studies have determined that class II MHC antigen expression was expressed on epithelial cells in 4 out of 5

patients (Norton, Al-Saffar, and Sloane 1992). Whereas no observable increase in ICAM-1, ELAM-1, or VCAM-1 staining occurred during hepatic GVHD.

Histopathologically, the 3 primary target organs of acute GVHD are characterized by the infiltration of lymphoid cells and epithelial destruction of cutaneous and mucosal barriers that facilitates the onset of lethal infections. Each of these organ systems demonstrates an increase in class II antigen expression and the numbers of $CD8^+$ or $T8^+$ cytotoxic T cells. In addition, cutaneous and intestinal GVHD depict elevated levels of IFN-γ and TNF-α as well as ICAM-1, VCAM-1 and ELAM-1 adhesion molecules. These common histopathologic and immunologic features of acute GVHD have established criteria for assessing the role of other potential GVHD-affected target organs.

Interstitial Pneumonitis and Lymphocytic Bronchiolitis/bronchitis

Interstitial pneumonitis. Interstitial pneumonitis (IP) has been recognized as a major cause of morbidity and mortality following BMT since the 1970's (Neiman et al. 1973, Meyers et al. 1975). A larger proportion of patients receiving allogeneic HLA-matched nonidentical bone marrow develop interstitial pneumonitis than the collective populations of autologous or syngeneic transplant patients (46% vs. 27%) (Crawford and Hackman 1993). Interstitial pneumonitis occurs with an incidence of 30-80% and results in a fatality rate of 50-60%. Although 60% of all interstitial pneumonitis occurs in conjunction with cytomegalovirus (CMV), 30-45% can not be attributed to bacterial or viral infection and is considered to be idiopathic (Meyers, Flournoy and Thomas 1982). Overall, interstitial pneumonitis represents the second leading cause of death in 100-day survivors other than the recurrence of leukemia (Wingard, Santos and Saral 1985). Clinically, the symptoms of interstitial pneumonitis include diffuse radiographic infiltrates, fever, dyspnea, non-productive cough, and hypoxemia (Meyers, Flournoy

and Thomas 1982, Wingard et al. 1988, Weiner et al. 1986). Histologically, IP is characterized as widespread perivascular mononuclear cell cuffing extending into the alveolar septa (Yousem et al. 1990).

Clinical data have defined the main predictive factors of IP following allogeneic BMT to be: (1) prior cytomegalovirus infection of either the donor or recipient, (2) dose or dose-rate of lung irradiation, (3) sex-matching of the donor and recipient, (4) pre-transplant conditioning chemotherapy, (5) dose of bone marrow cells, (6) number of transfusions prior to transplant, (7) granulocyte transfusions post transplant, (8) immunosuppressive therapy, and (9) age of recipient (Appelbaum et al. 1982, Bortin et al. 1982, Meyers, Flournoy and Thomas 1982, Sloane et al. 1983, Weiner et al. 1986, Neiman et al. 1977, Weiner and Dicke 1987, Breuer et al. 1988). Although clinical and experimental studies have failed to determine if IP occurs as a direct result of acute GVHD, compiled data has suggested that three types of IP exist following allogeneic BMT: (1) radiation-induced IP, (2) CMV-induced IP, and (3) idiopathic IP.

The effects of radiation on the lung have been documented since the early 1970's (Van Den Brenk 1971). The primary effects of radiation-induced lung injury occurs within the replicative mechanisms of pulmonary cells and reflects the proportion of cells irreparably damaged or killed resulting in depopulation. Cell damage and depopulation are responsible for the exudative, vascular and cicatricial changes known as radiation pneumonitis. The turnover rate of alveolar cells shows a direct correlation to the latent period that precedes the onset of radiation pneumonitis. Depopulation of alveolar cells reduces the production of alveolar surfactant and inhibits phagocytic capabilities. The loss of surfactant incurs osmotic collapse between the alveolar-capillary interface allowing the passage of blood transudates and hemorrhage into the alveolar lumen with excessive desquamation of damaged alveolar cells.

Consequently, instantaneous epithelial and endothelial damage occurs after irradiation that is not clinically apparent until after a "latent" period of 2 - 3 months (Rubin et al. 1980). In addition, the early decrease of type II lamellar bodies, increased cell turnover rates, pulmonary hemorrhage, and the presence of hyaline membrane disease often confuse the diagnosis of radiation pneumonitis with acute GVHD (Piguet et al. 1989, Wojno et al. 1994).

There is also a direct relationship between the onset of interstitial pneumonitis and dose as well as dose-rate of irradiation used for pre-transplant conditioning (Keane et al. 1981, Barrett, Depledge and Powles 1983, Ringdén et al. 1983). Both LA F_1 and CBA murine models have shown that IP was produced from the synergistic effects of irradiation and acute GVHD (Lehnert, Rybka and Seemayer 1986, Down et al. 1992). These experimental studies have shown that radiation alone produced a "pneumonitis" which may occur concurrently with GVHD-induced IP. Consequently, irradiation was not incorporated into the experimental protocols of this dissertation.

Cytomegalovirus infection remains an serious risk factor to the long-term survival and success of allogeneic BMT patients. It is most often detected when acute GVHD is present in grades II - IV, and incurs a high rate of mortality because no effective treatment is available. Seropositivity of either donor or host is one of the primary risk factors in developing CMV-induced IP (reviewed in Meyers et al. 1975, reviewed in Paulin et al. 1986).

The vast clinical and experimental literature on CMV is outside the scope of this dissertation. However, several studies have shown that an interdependence exists between the onset of CMV, acute GVHD and interstitial pneumonitis. Grundy et al. injected adult nonirradiated (B10 × B10.A) F_1 mice with murine CMV and a low dose of parental cells

(Grundy et al. 1985). The severity of acute GVHD was significantly increased despite the dose of parental lymphoid cells and IP was the prominent histopathological lesion observed within lung tissues. Clinical data have also shown that the simultaneous presence of acute GVHD and CMV significantly increased the incidence and mortality of IP (Neiman et al. 1973, 1976, 1977, Meyers, Flournoy and Thomas 1980, 1982, Miller et al. 1986, Cordonnier et al. 1986).

Further research has shown that acute murine CMV augments the ability of parental spleen cells to induce GVHD (Via et al. 1988). Nonirradiated (B10 × B10.A) F_1 mice were used to determine whether murine CMV infection produces a synergistic effect with GVHD through donor or host components. Donor mice inoculated with murine CMV 3 days prior to transplantation showed a reduced capacity to induce a GVHD-CMV associated IP, while murine CMV infection of the recipients 3 days prior to injection of parental cells exacerbated the development of IP. In addition, (B10 × B10.BR) F_1 mice injected with 20×10^6 B10.BR spleen cells and murine CMV developed a severe diffuse pneumonitis that was not observed with either spleen cells or CMV alone (Shanley et al. 1987). More than 80% of the cells obtained by BAL and characterized using multiparameter flow cytometric analysis were determined to be donor Thy 1.2^+ lymphocytes. In addition, 43% of BAL cells were $L3T4^+$ and 38% were Lyt 2^+. Consequently, an influx of donor T lymphocytes was observed during the development of IP as a result of GVHD and CMV.

Lymphocytic bronchiolitis/bronchitis. Lymphocytic bronchitis (LB) may also be a consequence of acute GVHD (Beschorner et al. 1978). Lymphocytic bronchitis was characterized by the moderate to marked homogenous infiltration of small, darkly stained lymphocytes within the bronchial mucosa and submucosa. The basement membrane often appeared vacuolated, and the mucosa showed single-cell necrosis, a loss of ciliated cells, and a

decrease in the number of goblet cells. Hyperplastic regenerative mucosa with large basophilic irregular nuclei are often present within the upper respiratory tract, and squamous metaplasia may be evident (Beschorner et al. 1978, Yousem et al. 1990). Clinically, LB becomes evident as bronchopneumonia during acute GVHD of grades II -IV. Although LB is a serious complication of acute GVHD, it is not associated with CMV infection or a high rate of mortality. Pulmonary LB appears similar to the lymphocytic infiltration that occurs in the lamina propria causing mucosal necrosis during intestinal GVHD.

The exacerbation of IP by acute GVHD and CMV infection, has confirmed the need to eliminate all traces of CMV from any protocol designed to assess pulmonary histopathology as a direct result of acute GVHD. Furthermore, clinical and experimental studies incorporating irradiation and/or CMV infection have failed to determine the immunopathology of acute GVHD-induced "idiopathic" IP. Consequently, the characterization of acute GVHD-induced idiopathic IP and the development of a successful treatment to alleviate or control its onset remain critical to the future success of allogeneic BMT.

CHAPTER III

FLOW CYTOMETRIC ANALYSIS OF PULMONARY BRONCHOALVEOLAR LAVAGE AND COLLAGENASE-DIGESTED CELL SUSPENSIONS THROUGHOUT ACUTE LETHAL GRAFT-VS.-HOST DISEASE

Introduction

Graft-vs.-Host Disease (GVHD), a frequent and often lethal complication of allogeneic bone marrow transplantation (BMT), develops when donor T lymphocytes initiate an immune response to disparate host major and minor histocompatibility antigens (Korngold and Sprent 1990). However, depletion of T lymphocytes from the donor inoculum often abrogates engraftment and increases the rate of leukemic relapse (Weiden et al. 1981, Tutschka et al. 1987, Korngold and Sprent 1990). The acute form of GVHD incurs a high incidence of mortality and diagnosis remains difficult due to the complicating effects of pre-transplant conditioning regimens. Therefore, defining the immunopathologic consequences of acute GVHD remains fundamental to the clinical management of allogeneic BMT patients.

Pulmonary complications are a significant cause of mortality during acute GVHD. Many studies have implicated interstitial pneumonitis and lymphocytic bronchitis as the prevalent histopathologic elements of acute GVHD (Neiman et al. 1977, Beschorner et al. 1978, Sloane et al. 1983, Sullivan et al. 1986, Atkinson et al. 1991, Sloane and Norton 1993), but research has not determined a correlation between the onset of these histopathologies or their involvement throughout the development of a pulmonary syndrome that may be the direct result of acute GVHD. Previous studies have had difficulties discerning the initial cause of these complications due to the use of chemotherapeutic agents, irradiation, immunosuppressive drugs and overt infection. As a result, idiopathic interstitial pneumonitis has been implicated as a

direct consequence of acute GVHD but conclusive data has not been documented.

Bronchoalveolar lavage (BAL) is an invasive procedure commonly used to access the cells and non-cellular components present on the epithelial surface of alveoli that are representative of ongoing inflammatory and immunological responses within the lower respiratory tract (Hunninghake et al. 1979, Reviewed by Reynolds 1987). Prior to the availability of monoclonal antibodies, differential counts of Wright-Giemsa-stained cytocentrifuge preparations provided the only means available to determine the phenotypes of cells within BAL samples. Today, BAL is routinely used in an attempt to diagnosis pulmonary infection, pathogenesis, allograft rejection and following bone marrow transplantation. Consequently, BAL and collagenase-digestion were used to determine if any variations in alveolar or parenchymal populations occurred throughout acute GVHD. Experimentally, BAL is used in conjunction with collagenase-digestion to produce mononuclear cell suspensions from pulmonary tissues.

In the present study, BAL and collagenase-digestion were conducted according to standardized methods outlined for obtaining the optimal number of viable mononuclear cells from normal rat lung tissues (Holt 1979, Holt et al. 1985). BAL procedures were conducted prior to collagenase-digestion on adult nonirradiated (DA × LEW) F_1 hybrid rats in the absence of chemotherapy, irradiation, immunosuppression and overt infection to detect phenotypic changes within alveolar and parenchymal compartments throughout acute lethal GVHD. BAL samples and collagenase-digests from control and GVHD-induced rats were incubated with a wide panel of mouse anti-rat monoclonal antibodies.

Although previous studies quantified the results of immunoperoxidase staining by manually counting 300 - 500 labeled BAL and collagenase-digested cells (Holt and Schon-

Hegrad 1987), the use of flow cytometric analysis (FACS) analysis has provided a quantitative method to measure phenotypic variations. Consequently, FACS analysis was used to determine if phenotypic variations occurred within BAL and collagenase-digests throughout acute GVHD.

Materials and Methods

Animals

Lewis (LEW:RT^{1}), DA (RT1^{a}) and (DA×LEW) F_1 hybrid rats were bred in the animal care facility at Loyola University Stritch School of Medicine. Acute, lethal GVHD was induced in 8 - 12 week old nonirradiated (DA × LEW) F_1 rats by the intravenous injection of 1×10^6 DA lymphoid (spleen and lymph node) cells/gram body weight as previously described (Clancy and Mauser 1981). All F_1 animals injected with parental DA lymphoid cells developed classic signs of acute systemic GVHD by day 14, and 100% morbidity as well as mortality by day 21.

To establish a control group, the lungs were excised on days 3 and 7 from 4 (2 animals per time point) noninjected F_1 rats, 4 GVHD-induced F_1 rats, and 4 F_1 animals intravenously injected with 1×10^6 F_1 lymphoid cells/gram body weight. All F_1 animals were matched to litter and sex. Histological and quantitative data confirmed that there were no differences between noninjected and syngeneically-injected F_1 animals compared on days 3 and 7. In addition, the pulmonary histopathology present by day 7 in GVHD-induced F_1 animals was absent in both noninjected and syngeneically-injected F_1 groups. Therefore, noninjected adult, nonirradiated F_1 hybrid rats were used as controls throughout this study.

Noninjected F_1 control and GVHD-induced animals were killed by ether overdose. GVHD animals were killed on days 3, 7, 10, 14, and 15 - 21 (5 animals per time point)

following injection. GVHD animals were matched to each other and corresponding controls by litter and sex when possible. All animal manipulations were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago.

Lung Preparation

One control and one GVHD-induced F_1 rat were killed for BAL and collagenase digestion per surgical session. The rats were placed ventral side up on a small metal rack tilted at a 45° angle within an 8 × 10 pyrex container. Suture (3.0) was tied around each front limb at the distal joint and then to the metal rack. An incision was made horizontally along the distal edge of the diaphragm, then from the diaphragmatic border through the rib cage to the clavicle on each side, and horizontally from clavicle to clavicle. The frontal chest plate consisting of ribs and sternum was carefully removed so as not to disturb any thoracic organs. The descending aorta was severed above the diaphragm and a 50 cc syringe filled with PBS chilled to 4° C was attached to a 22 gauge butterfly needle placed within the right atrium. PBS was injected until the pulmonary vasculature was flushed and the lung tissue turned white.

Bronchoalveolar Lavage

After perfusion with PBS, the lungs with heart and trachea attached were removed from the rat by the distal end of the trachea. A 16 gauge needle was inserted approximately 2" into one end of a 3" length of 3 mm dialysis tubing. The other end of the tubing was cut on a 45° angle. This tubing-needle apparatus provided an endotracheal device that was inserted within the trachea until the beveled end of the plastic tubing was just above the tracheal bifurcation. The endotracheal device was tied in place using 3.0 cuticle suture. The lungs, heart, and inserted endotracheal device were placed within a small clear plastic specimen bag with a ziploc edge. The ziploc edge was sealed around the protruding endotracheal device, and

the ziploc bag, containing the lungs, was placed within the circular depression of a poached egg tray and angled at 45° within the 8 × 10 pyrex container. The lung-filled ziploc bag was anchored to the plastic poached egg tray using hemostats. A 10 cc syringe was filled with Hanks Balanced Salt Solution (HBSS) warmed to 37° C containing 0.4% Lidocaine as previously described (Rabinovitch and DeStefano 1976, Holt 1979). The syringe was attached to the endotracheal device and 10 cc of HBSS/Lidocaine was slowly injected until the lungs were fully expanded but without any surface leakage. The HBSS/Lidocaine was kept within the lung for 2 minute increments before the syringe was detached and the fluid allowed to drain by gravitational force into 50 cc conical tubes. The lungs were not manipulated by hand at any time. This process was repeated until 100 cc of HBSS/Lidocaine had been injected and the subsequent BAL fluid was collected.

After BAL procedures were completed, the lungs were removed from the ziploc bags and the heart as well as bronchi were discarded. The BAL fluid was layered over Lymphocyte Separation Medium ® and centrifuged for 30 minutes at 1800 RPM. The buffy coat was removed after centrifugation and washed 3 times in HBSS.

Homogenization vs. Collagenase Digestion

To ensure that collagenase-digestion provided optimal cell yields, experiments were conducted to determine the difference between homogenization and collagenase-digestion to obtain viable cell suspensions from spleens of F_1 rats. Another preliminary experiment used various lots of secondary mouse anti-rat IgG Fab^2 fragments conjugated to FITC on homogenized as well as collagenase-digested spleen, collagenase-digested lung, and BAL cell suspensions to assess the number of cells obtained as well as mean fluorescence intensity (MFI) (Table 1). An additional experiment was conducted using propidium iodide to remove

dead cells during FACS analysis (Table 2). Further preliminary studies labled collagenase-digested spleen and homogenized spleen tissues with 5 monoclonal mouse anti-rat antibodies to demonstrate that collagenase-digestion was an optimal technique for obtaining parenchymal lung cell suspensions for FACS analysis (Tables 3 and 4). The data provided from these 4 assays determined that collagenase-digestion in conjunction with propidium iodide assured an optimal number of viable cells as well as receptors, and appeared more effective than homogenization.

Collagenase digestion

The whole lung lobes were blotted dry, weighed and separated into .9 g increments that were sliced into 1mm thick sections using a razor blade on dental wax. The sectioned lungs were placed into small glass mixing flasks and plastic coated stirring rods that had previously been cleaned with an ammonium hydroxide/ethanol solution for 24 hours prior to coating each flask and stirring rod with liquid Silicone (Sigma). The .9 grams of sliced lung tissue was placed into a siliconized flask with 32.43 mg of Type I Collagenase (150 U/ml), 43 µl of DNAse (50 U/ml), and 30 ml of PBS. The flasks were placed on stir plates within an incubator at 37^0 C for 90 minutes. After collagenase digestion was completed the mixture was layered over Lymphocyte Separation Medium ® and centrifuged at 1800 RPM for 30 minutes. The buffy coat was removed and washed 3 times in HBSS. The BAL and collagenase-digested cell suspensions were counted using Trypan Blue Exclusion, resuspended at a concentration of 1×10^6 cells/ml chilled to 4° C. Each flask and stirring rod was cleaned overnight with an ammonium hydroxide/ethanol mixture and re-siliconized the next day.

Antibody Labeling for Flow Cytometric Analysis

All the antibodies used for labeling (Table 5) were diluted in PBS with 5% fetal calf

serum (FCS) to a final concentration of 1:100. One hundred microliters of BAL and collagenase-digested cell suspensions were added to individual wells of a microtiter plate. One hundred microliters of each antibody were added to corresponding rows of cell suspensions. One row received only 100 µl of PBS to serve as a control for nonspecific staining. The mononuclear cell/antibody suspensions were incubated for one hour at 4^0 C. After incubation, the plate was washed 3 times in PBS to prepare for addition of 2° antibody. Fab^2 fragments of mouse anti-rat IgG conjugated to fluorescein (Cappel, Organo Technic Corp., Westchester, PA) noted as heavy and light chain specific was used as 2° antibody. One hundred microliters of 2° antibody diluted in PBS to a final concentration of 1:450 were added to each well and incubated for one hour at 4° C. After incubation, the plate was washed 3 times in PBS, and 100 µl propidium iodide diluted in PBS to a final concentration of 1:100 were added to each well of the plate just prior to FACS analysis.

Results

Body Weight, Lung Weight, Lung Index

Body weights, lung weights and lung indices were calculated for all control and GVHD animals (Tables 6 - 8). All animals were matched to sex and age when possible. Lung indices were calculated using the formula for spleen index (Simonsen 1962). The average lung index for day 7 (N = 3) was 1.47, for day 10 (N = 1) was 1.5, and for day 14 (N = 3) was 1.81. The percent difference between averaged day 7 and day 14 lung indices demonstrated an increase of 23.13 % which reflected a decrease in body weight. Lung weights averaged 2.7 g regardless of sex or weight differences.

Cell Yields From Parenchymal Collagenase Digests and BAL Fluid

Although lung weights remained similar regardless of sex or weight differences, large fluctuations occurred within the cell yields obtained from day 7 collagenase digests.

Control male rats consistently produced more viable mononuclear cells from collagenase-digested tissues as well as BAL samples on days 7 and 10 then corresponding female GVHD animals (Tables 9 - 12). However, cell yields obtained from the collagenase-digestion of female GVHD animals on day 14 were higher than control values regardless of sex (Table 13). BAL samples from the same animals did not show a consistent trend (Table 14). There may be a direct correlation between relative increases or decreases within BAL and collagenase-digested cell yields. An increased number of harvested BAL cells appear to accompany decreased parenchymal yields, while decreased BAL yields correspond to an increased number of parenchymal cells.

FACS Analysis of Parenchymal and BAL Cells

The initial testing of collagenase-digests and BAL cells was conducted using a final concentration of 2° antibody at 1:150. However, this dilution provided an excess of non-specific staining and was retested to determine an optimal concentration of 1:450. Although each monoclonal antibody labeled pulmonary BAL or collagenase-digested cell suspensions, at no time were the values obtained from FACS analysis consistent among control or GVHD animals (Tables 5, 15 - 19).

Discussion

FACS analysis of was used to assess the phenotypic variations within BAL and collagenase-digested cell suspensions taken from adult nonirradiated (DA × LEW) F_1 hybrid rats throughout acute GVHD. Although the number of viable gated cells present within GVHD populations differed from those present in control cell suspensions, data were inconsistent for all animals and time points tested. However, there appeared to be a direct relationship between the number of cells obtained via BAL and collagenase-digestion. This correlation suggested that the process of BAL did not only recover mononuclear cells from the alveolar space, but

also harvested mononuclear cells from parenchymal tissues. Consequently, our results demonstrated that neither antibody-labeled BAL fluid nor collagenase-digested cell suspensions were capable of defining the onset, time course, anatomical location or severity of GVHD-induced pulmonary histopathology. Therefore, the present study was extended to include histological and immunohistochemical analysis.

Other reports comparing immunohistochemical analysis of transbronchial biopsies to FACS analysized BAL fluid support our conclusions. These studies indicate that BAL alone may be used to detect non-specific pathology or infection, although a combination of BAL and transbronchial biopsy provided more accurate results (Reviewed by Heurlin et al. 1989). In addition, BAL can not be relied upon to diagnose grades of allograft rejection or acute GVHD (Milburn et al. 1988, Leskinen et al. 1990, Reviewed by Shennib and Nguyen 1991).

TABLE 1

EFFICACY OF HOMOGENIZATION VS. COLLAGENASE DIGESTION AS DETERMINED BY USING DIFFERENT LOTS OF SECONDARY ANTIBODY

Cell Suspensions	Percent Gated Cells	Mean Fluorescence Intensity
Homogenized Spleen (O)	2.1	92.44
Homogenized Spleen (M)	3.7	118.68
Homogenized Spleen (N)	6.9	485.34
Collagenized Spleen (N)	9.4	261.99
Collagenized Lung (N)	12.6	214.73
BAL (N)	7.0	412.36

Old Secondary Antibody Staining with High Background (O)
Secondary Antibody Obtained from Dr. Schneider (M)
Replacement Secondary Antibody from Cappel (N)

TABLE 2

EFFICACY OF HOMOGENIZATION VS. COLLAGENASE DIGESTION IN COMBINATION WITH PROPIDIUM IODIDE

Cell Suspensions	Percent Gated Cells	Mean Fluorescence Intensity	Propidium Iodide Average for 5 Antibodies
Homogenized Spleen (O)	12.6	835.46	15.1
Homogenized Spleen (M)	13.6	828.52	15.2
Homogenized Spleen (N)	14.0	823.37	12.5
Collagenized Spleen (N)	15.1	781.63	17.0
Collagenized Lung (N)	30.2	835.22	32.2
BAL (N)	35.3	817.89	36.6

Old Secondary Antibody Staining with High Background (O)
Secondary Antibody Obtained from Dr. Schneider (M)
Replacement Secondary Antibody from Cappel (N)

TABLE 3

EFFICACY OF HOMOGENIZATION VS. COLLAGENASE DIGESTION AS DETERMINED BY PRELIMINARY FLOW CYTOMETRIC ANALYSIS

Cell Suspensions	OX8		OX12		OX19		W3/25		ED1	
	% Gated	MFI	% Gated	MFI	% Gated	MFI	% Gated	MFI	% Gated	MFI
Homogenized Spleen	26.2	198.48	37.4	232.11	62.3	152.89	43.8	120.91	11.6	220.67
Collagenized Spleen	36.0	176.26	44.8	191.61	70.0	127.84	53.3	101.55	53.9	114.03
Collgenized Lung	47.2	159.02	30.4	164.46	59.5	130.32	53.9	114.03	23.9	157.60
BAL	12.4	412.36	33.5	183.64	14.8	314.29	13.9	271.78	18.9	294.29

% Gated = Percent Gated Viable Cell
MFI = Mean Fluorescence Intensity

TABLE 4

EFFICACY OF HOMOGENIZATION VS. COLLAGENASE DIGESTION AS DETERMINED BY PERCENT GATED DIFFERENCE AND MEAN FLUORESCENCE INTENSITY

Antibody-Labeled Spleen Cells	Percent Gated Increase	Mean Fluorescence Intensity Decrease
IgG only	27	46
OX8	28	12
OX12	17	18
OX19	11	17
W3/25	18	16
ED1	21	52

TABLE 5

FLOW CYTOMETRIC ANALYSIS OF CONTROL AND DAY 7 GVHD COLLAGENASE-DIGESTED CELL SUSPENSIONS

Antibody	5/16/91	7/9/91		8/6/91		8/27/91	
	C	C	G	C	G	C	G
	Dilution 1:150			Dilution 1:450			
IgG	12.6	28.4	41.3	1.0	2.2	4.8	5.9
	Dilution 1:100						
MOPC10				5.9	1.3	5.2	7.3
UPC21				5.1	1.5	5.0	7.1
OX6		62.0	75.6	30.1	29.8	39.8	44.1
OX8	47.2	60.0	66.0	51.2	23.2	39.6	32.4
OX12	30.4	47.5	53.6	24.8	21.7	27.6	34.0
OX19/52	59.5	29.3	73.9	54.0	38.9	40.9	48.9
OX39		44.8	55.0	11.6	6.2	14.2	21.1
OX41						36.3	44.7
OX42		60.0	69.2	36.5	31.8	36.9	46.9
W3/25	53.9	58.1	72.7	44.7	36.5	40.4	58.8
ED1	23.9	38.4	65.6	37.2	24.3	33.0	27.2
ED2			43.7	7.9	3.7	6.2	11.8

C = Control Rats
G = GVHD-Induced Rats

TABLE 6

BODY WEIGHT, LUNG WEIGHT AND LUNG INDEX OF CONTROL AND DAY 7 GVHD F_1 HYBRID RATS

	7/9/91		8/6/91		8/27/91	
	C (Female)	G (Female)	C (Male)	G (Female)	C (Male)	G (Female)
Body Weight (g)	238.7	233.6	368.8	224.5	378.0	203.0
Lung Weight (g)	2.8	2.5	2.7	2.8	2.9	2.9
Lung Index	0.92		1.64		1.84	
Date of Birth	2/19/91		4/9/91		6/2/91	

C = Control Rats
G = GVHD-Induced Rats

TABLE 7

BODY WEIGHT, LUNG WEIGHT AND LUNG INDEX
OF CONTROL AND DAY 10 GVHD
F_1 HYBRID RATS

	8/8/91	
	C (Male)	G (Female)
Body Weight (g)	359.0	204.0
Lung Weight (g)	2.9	2.4
Lung Index	1.5	
Date of Birth	5/13/91	

C = Control Rats
G = GVHD-Induced Rats

TABLE 8

BODY WEIGHT, LUNG WEIGHT AND LUNG INDEX OF CONTROL AND DAY 14 GVHD F_1 HYBRID RATS

	7/16/91		8/13/91		9/3/91	
	C (Male)	G (Female)	C (Male)	G (Female)	C (Male)	G (Female)
Body Weight (g)	235.0	188.0	361.0	193.5	360.0	163.7
Lung Weight (g)	2.1	2.7	3.0	2.9	2.8	2.7
Lung Index	1.55		1.88		2.00	
Date of Birth	2/19/91		5/13/91		6/2/91	

C = Control Rats
G = GVHD-Induced Rats

TABLE 9

CELL YIELDS OF CONTROL AND DAY 7 GVHD COLLAGENASE-DIGESTED CELL SUSPENSIONS

	7/9/91		8/6/91		8/27/91	
	C (Female)	G (Female)	C (Male)	G (Female)	C (Male)	G (Female)
Cell Yields (Millions)	4.0	8.0	27.6	16.5	17.8	16.0

C = Control Rats
G = GVHD-Induced Rats

TABLE 10

CELL YIELDS OF CONTROL AND DAY 7 GVHD BRONCHOALVEOLAR LAVAGE CELL SUSPENSIONS

	7/9/91		8/6/91		8/27/91	
	C (Female)	G (Female)	C (Male)	G (Female)	C (Male)	G (Female)
Cell Yields (Millions)	2.0	2.8	3.6	2.8	11.7	2.2

C = Control Rats
G = GVHD-Induced Rats

TABLE 11

CELL YIELDS OF CONTROL AND DAY 10 GVHD
COLLAGENASE-DIGESTED
CELL SUSPENSIONS

	8/8/91	
	C (Male)	G (Female)
Cell Yields (Millions)	11.6	8.6

C = Control Rats
G = GVHD-Induced Rats

TABLE 12

CELL YIELDS OF CONTROL AND DAY 10 GVHD
BRONCHOALVEOLAR LAVAGE
CELL SUSPENSIONS

	8/8/91	
	C (Male)	G (Female)
Cell Yields (Millions)	6.8	2.9

C = Control Rats
G = GVHD-Induced Rats

TABLE 13

CELL YIELDS OF CONTROL AND DAY 14 GVHD COLLAGENASE-DIGESTED CELL SUSPENSIONS

	7/16/91		8/13/91		9/3/91	
	C (Female)	G (Female)	C (Male)	G (Female)	C (Male)	G (Female)
Cell Yields (Millions)	7.5	28.2	30.8	51.1	45.3	50.4

C = Control Rats
G = GVHD-Induced Rats

TABLE 14

CELL YIELDS OF CONTROL AND DAY 14 GVHD BRONCHOALVEOLAR LAVAGE CELL SUSPENSIONS

	7/16/91		8/13/91		9/3/91	
	C (Female)	G (Female)	C (Male)	G (Female)	C (Male)	G (Female)
Cell Yields (Millions)	2.4	2.7	9.2	5.1	1.7	5.4

C = Control Rats
G = GVHD-Induced Rats

TABLE 15

FLOW CYTOMETRIC ANALYSIS OF CONTROL AND DAY 7 GVHD BRONCHOALVEOLAR LAVAGE CELL SUSPENSIONS

Antibody	5/16/91	7/9/91		8/6/91		8/27/91	
	C	C	G	C	G	C	G
	Dilution 1:150			Dilution 1:450			
IgG	7.0	19.5	19.7	7.3	7.1	12.5	9.8
	Dilution 1:100						
MOPC10				6.4	7.7	12.1	
UPC21				6.7	11.9	11.3	
OX6		34.6	47.3	11.8	24.1	32.9	20.9
OX8	12.4	25.2	31.1	13.1	26.8	18.3	16.8
OX12	33.5	49.6	50.1	15.6	31.9	40.4	29.4
OX19/52	14.8	28.5	38.6	17.9	20.3	25.5	5.0
OX39		19.2	23.7	11.0	12.2	16.3	2.6
OX41						63.9	67.0
OX42			37.1	22.0	32.5	35.2	28.6
W3/25	13.9		32.0	17.1	19.3	29.6	8.0
ED1	18.9		39.3	16.5	25.8	23.7	18.4
ED2				6.9	9.5	12.1	8.6

C = Control Rats
G = GVHD-Induced Rats

TABLE 16

FLOW CYTOMETRIC ANALYSIS OF CONTROL AND DAY 10 GVHD COLLAGENASE-DIGESTED CELL SUSPENSIONS

Antibody	8/9/91	
	C	G
	Dilution 1:450	
IgG	15.3	9.7
	Dilution 1:100	
MOPC10	16.6	8.9
UPC21	15.8	12.1
OX6	46.6	50.8
OX8	52.4	40.5
OX12	34.7	41.8
OX19/52	57.7	55.4
OX39	28.3	29.4
OX41		
OX42	53.4	58.3
W3/25	58.0	63.0
ED1	49.1	43.9
ED2	20.2	17.8

C = Control Rats
G = GVHD-Induced Rats

TABLE 17

FLOW CYTOMETRIC ANALYSIS OF CONTROL AND DAY 10 GVHD BRONCHOALVEOLAR LAVAGE CELL SUSPENSIONS

Antibody	8/9/91	
	C	G
	Dilution 1:450	
IgG	21.5	7.4
	Dilution 1:100	
MOPC10	23.9	11.3
UPC21	23.5	17.7
OX6	45.3	26.1
OX8	34.3	34.3
OX12	51.2	28.4
OX19/52	50.3	59.1
OX39	30.7	20.7
OX41		
OX42	54.2	30.1
W3/25	49.4	48.7
ED1	42.6	43.7
ED2	23.1	14.0

C = Control Rats
G = GVHD-Induced Rats

TABLE 18

FLOW CYTOMETRIC ANALYSIS OF CONTROL AND DAY 14 GVHD COLLAGENASE-DIGESTED CELL SUSPENSIONS

Antibody	7/16/91		8/13/91		9/4/91	
	C	G	C	G	C	G
	Dilution 1:150		Dilution 1:450			
IgG	18.9	23.5	3.7	2.4	12.7	6.6
	Dilution 1:100					
MOPC10			3.6	3.0	13.6	6.6
UPC21			2.9	2.2	13.0	7.9
OX6	39.3	86.0	30.3	58.8	45.3	77.1
OX8	59.7	44.4	33.2	34.5	46.0	31.0
OX12	35.3	36.8	26.2	23.9	39.7	24.0
OX19/52	60.6	86.1	34.0	58.5	50.1	78.5
OX39		49.5	9.9	9.7	23.9	29.0
OX41					48.4	28.2
OX42		45.9	25.4	30.1	46.2	32.8
W3/25	53.9	84.7	30.9	58.9	47.6	80.7
ED1	23.9	82.2	18.5	33.8	40.7	63.4
ED2		55.7	5.9	9.5		

C = Control Rats
G = GVHD-Induced Rats

TABLE 19

FLOW CYTOMETRIC ANALYSIS OF CONTROL AND DAY 14 GVHD BRONCHOALVEOLAR LAVAGE CELL SUSPENSIONS

Antibody	7/16/91		8/13/91		9/4/91	
	C	G	C	G	C	G
	Dilution 1:150		Dilution 1:450			
IgG	10.4	8.5	6.0	3.9	7.4	17.1
	Dilution 1:100					
MOPC10			6.5	4.5	7.0	17.2
UPC21			5.8	3.7	7.5	16.2
OX6	21.3	27.6	26.1	27.8	23.8	53.6
OX8	14.5	12.4	11.1	13.4	13.7	28.3
OX12	21.4	45.5	26.3	30.3	28.5	38.2
OX19/52	23.3	16.3	14.2	25.0	20.9	53.7
OX39		9.6	16.6	6.2	9.1	24.4
OX41					67.6	41.3
OX42	29.1	39.6	26.3	32.6	31.7	46.8
W3/25	20.4	16.0	13.7	32.0	22.9	57.8
ED1	25.6	23.0	14.6	29.6	18.4	44.2
ED2			6.0	7.9		

C = Control Rats
G = GVHD-Induced Rats

CHAPTER IV

INTERSTITIAL PNEUMONITIS AND LYMPHOCYTIC BRONCHIOLITIS/BRONCHITIS AS A DIRECT RESULT OF ACUTE LETHAL GRAFT-VS.-HOST DISEASE DUPLICATE THE HISTOPATHOLOGY OF LUNG ALLOGRAFT REJECTION

Abstract

Pulmonary complications are often lethal components of acute Graft-vs.-Host Disease (GVHD). Although interstitial pneumonitis and lymphocytic bronchitis have been implicated as elements of acute GVHD, previous studies have not determined a correlation between the onset of these histopathologies or their contribution to a pulmonary syndrome that may occur as a direct result of acute GVHD. The present study used the adult, nonirradiated (DA × LEW) F_1 hybrid rat in the absence of chemotherapy, immunosuppressive drugs or overt infection to study these aspects of pulmonary pathology during acute GVHD. F_1 animals were intravenously injected with 1×10^6 DA parental lymphoid cells/gram body weight which produced 100% morbidity and mortality by day 21. Neither syngeneically injected nor noninjected F_1 control animals contained any observable or measurable histopathology. In addition, GVHD and control tissues did not contain bacterial, fungal, or CMV contamination as determined by specific tissue and immunohistochemical staining. GVHD animals were killed on days 3, 7, 10, 14, and 15 - 21 following injection. Four micron, whole lobe tissue sections were stained with H&E, and histologic alterations within predetermined tissue sites were quantified using light-microscopic Image Analysis. Alveolar septal widths and perivascular infiltrate volume densities were increased significantly above controls by day 7, and reached 2.4 and 2.6 fold increases respectively by day 21. This data corroborated the development of an interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis that duplicated the histopathology of lung allograft rejection. The discovery of pulmonary pathology corresponding to lung allograft rejection during

acute GVHD in the adult F_1 rat implicates the lung as a potential target organ.

Introduction

Graft-vs-Host Disease (GVHD), a frequent consequence of allogeneic bone marrow transplantation (BMT), develops when donor T lymphocytes initiate an immune response to disparate host major and minor histocompatibility antigens (Korngold and Sprent 1990). However, the depletion of T lymphocytes from the donor inoculum often abrogates engraftment (Korngold and Sprent 1990). Therefore, defining the immunopathologic consequences of acute GVHD is fundamental to the clinical management of allogeneic BMT patients.

The pulmonary complications of GVHD are consistently associated with an increase in morbidity and mortality. However, the initiation, progression and components of acute GVHD within the lung remain ill-defined and controversial. Many studies have implicated interstitial pneumonitis and lymphocytic bronchitis as prevalent elements during acute GVHD (Atkinson et al. 1991, Sloane and Norton 1993, Sloane et al. 1983, Beschorner et al. 1978, Neiman et al. 1977, Sullivan et al. 1986, Stein-Streilein et al. 1981), but these studies have not determined a correlation between the onset of these histopathologies or their involvement throughout the development of a pulmonary syndrome that may be the direct result of acute GVHD. In contrast, several well-defined respiratory disorders are considered clinical manifestations of chronic GVHD (Stein-Streilein et al. 1981, Bradstock et al. 1984, Holland et al. 1988, Roca et al. 1982, Ralph et al. 1982, Atkinson et al. 1984, Atkinson et al. 1989, Wyatt et al. 1984, Raschko et al. 1989). Some studies suggest that interstitial pneumonitis is characteristic of chronic GVHD (Sullivan et al. 1986, Piguet et al. 1989, Perreault et al. 1985, Wingard, Santos and Saral 1985). However, interstitial pneumonitis associated with chronic pulmonary disease is accompanied by fibrotic changes that are absent throughout the acute phase of GVHD.

We have used the adult, nonirradiated (DA × LEW) F_1 hybrid rat (Clancy and Mauser 1981) to investigate the possibilities that: (1) a pulmonary syndrome which duplicates the histopathology of lung allograft rejection develops as a direct result of acute GVHD, (2) interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis are integral components of this syndrome, and (3) the lung is an important target organ of acute GVHD. This animal model, based on the injection of parental lymphoid cells into F_1 adults, consistently produced a systemic GVHD that paralleled the sequential organ involvement observed in allogeneic BMT patients. In addition, this model ruled out the influence of irradiation, chemotherapy, immunosuppression, and overt infection. Although irradiation has been incorporated into the majority of animal studies investigating GVHD, the present nonirradiated model omitted the primary lesions of epithelial and endothelial damage that accompany irradiation prior to experimentation (Rubin et al. 1980, Law, Ahier and Coultas 1986, Gross 1977, Travis 1980, Phillips 1966, Down 1986, Keane, Van Dyk and Rider 1981, Barrett, Depledge and Powles 1983, Kurohara and Casarett 1972, Van Den Brenk 1971), as well as the exacerbation of GVHD following irradiation (Down et al. 1992, Lehnert, Rybka and Seemayer 1986, Meyers, Flournoy and Thomas 1982).

Materials and Methods

Animals

Lewis (LEW:RT^1), DA ($RT1^a$) and (DA×LEW) F_1 hybrid rats were bred in the animal care facility at Loyola University Stritch School of Medicine. Acute, lethal GVHD was induced in 8 - 12 week old nonirradiated (DA × LEW) F_1 rats by the intravenous injection of 1×10^6 DA lymphoid (spleen and lymph node) cells/gram body weight as previously described (Clancy and Mauser 1981). All F_1 animals injected with parental DA lymphoid cells developed classic signs of acute systemic GVHD by day 14, and 100% morbidity as well as mortality by day 21.

To establish a control group, the lungs were excised on days 3 and 7 from 4 (2 animals per time point) noninjected F_1 rats, 4 GVHD-induced F_1 rats, and 4 F_1 animals intravenously injected with 1×10^6 F_1 lymphoid cells/gram body weight. All F_1 animals were matched to litter and sex. Histological and quantitative data confirmed that there were no differences between noninjected and syngeneically-injected F_1 animals compared on days 3 and 7. In addition, the pulmonary histopathology present by day 7 in GVHD-induced F_1 animals was absent in both noninjected and syngeneically-injected F_1 groups. Therefore, noninjected adult, nonirradiated F_1 hybrid rats were used as controls throughout this study.

Noninjected F_1 control and GVHD-induced animals were killed by ether overdose. GVHD animals were killed on days 3, 7, 10, 14, and 15 - 21 (6 animals per time point) following injection. GVHD animals were matched to each other and corresponding controls by litter and sex when possible. All animal manipulations were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago.

Histology

The heart, lungs and trachea were removed intact and individual lobes were severed from the right mainstem bronchi. The left lung lobe was used for immunohistochemical analysis. Each right lung lobe was placed in 10% buffered formalin. Whole spleens and kidneys were dissected as controls for histological preparation and staining.

All tissues were prepared for routine paraffin sectioning. Each lobe was sectioned at 4 μm along an anterior-posterior frontal plane at three differing tissue layers. A minimum of 20 floating sections per tissue block were mounted on Superfrost Plus slides (Fisher, Pittsburgh, PA). Four micron, frontal anterior-posterior sections of spleens and kidneys were also

prepared.

In addition to H&E staining, all tissues were tested with Brown-Brenn Gram and Grocotts Methenamine Silver stains to detect the presence of bacteria and fungi, respectively. Immunohistochemical staining for cytomegalovirus was conducted using a combination of two monoclonal mouse anti-human antibodies, DDG9 and CCH2 (Dako, Carpenteria, CA) (Wirgart et al. 1990, Niedobitek et al. 1988). Tissues were randomly selected from animals killed on days 0 (control), as well as 7, 14, and 18 days following injection to rule out the presence of infection. All tissues were devoid of viral, fungal or bacterial contamination.

Grading of Histopathology

The grading system used throughout this study followed previously published guidelines used to classify the severity and progression of lung allograft rejection (Yousem et al. 1990).

Image Analysis

Image analysis was accomplished using NIH Image installed on an Apple IIcx computer interfaced to a Leitz (Optische Werke; Wetzlar, Germany) microscope using a Scion Image Capture Board (Scion Corp.; Frederick, MD) and a MOS Javelin Solid State video camera (Javelin Electronics; Torrance, CA). All images were digitized at a magnification of 25X. Prior to each session, the microscope lamp was turned on for 30 minutes to stabilize the illumination source. Uniformity of the optical system was enhanced by automatically subtracting a blank field from a part of the slide not containing tissue with the illumination adjusted to an average pixel value of 127 on the Look-Up-Table (LUT). The optical density corresponding to specific tissue components was 148 - 238 on the LUT as determined by preliminary analyses of

random sections.

A grid was designed to provide random, systematically reproducible tissue sites for quantification by image analysis. The grid pattern was superimposed on the center of each H&E-stained lung section. Points along the grid pattern were 80 μm apart and covered the entire tissue section without producing points outside the borders of the section. If a grid site did not contain measurable tissue it was not used.

A total of 36 animals (6 animals per time point) was used in this study. Five random, systematically reproducible tissue sites were measured per tissue section, using the grid and selection criteria described above. To quantitate alveolar septal width, 6 equidistant length measurements perpendicular to the long axis were taken along the entire selected alveolar septae. To quantitate the volume density of perivascular infiltrates, circumferential and area measurements were obtained for the lumen, intima, and adventitial limiting plate of selected pulmonary vessels. The final volume density of the adventitial space was based on the optical density of perivascular infiltration as calculated by the LUT values described above.

Mean values of the measurements obtained for alveolar septal width and perivascular infiltrate volume density were calculated for each tissue section. The final means from each animal were grouped according to the parameter measured and time point tested. Differences between the overall, cumulative means derived from each parameter and time point were tested for statistical significance using a one-way Analysis of Variance and Tukey post-hoc analysis (Spatz and Johnston 1984).

Results

Histology

Neither syngeneically-injected F_1 nor noninjected F_1 control tissues contained any observable or measurable histopathology. Day 3 GVHD lung sections were also normal (data not shown). Therefore, all control and day 3 GVHD animals corresponded to Grade 0 lung allograft rejection criteria (Yousem et al. 1990). The alveolar septae at both time points were normal in width and cellularity. Alveoli were free of hemorrhage, cellular debris and elevated numbers of macrophages. The perivascular adventitial space was delineated by an intact limiting plate (Yousem et al. 1992), and filled with loose connective tissue that contained minimal cellularity. Bronchiolar smooth muscle and epithelium were normal, and were not surrounded or infiltrated by lymphocytes. Figure 1A represents a venule characteristic of control lung tissues. Figure 1B represents alveolar septae characteristic of control lung tissues, and contains examples of alveolar septae chosen for quantification by image analysis.

Day 7 GVHD tissue sections were characterized by the presence of infrequent perivascular mononuclear infiltrates that duplicated Grade 1 (Minimal acute rejection) (Yousem et al. 1990) allograft rejection criteria. Sites of perivascular infiltration were peripherally located throughout approximately 60% of each whole lung lobe tissue section, but were not obvious at low magnification. Both venules and arterioles were infiltrated with mononuclear cells, although the majority of affected vessels at day 7 were venules. Affected vascular adventitial spaces were infiltrated by small, round mononuclear cells layered 2-3 cells thick that appeared to migrate toward the adventitial limiting plate (Yousem et al. 1992). By day 7, the majority of intra-luminal mononuclear cells demonstrated an apparent affinity for, and adherence to, the vascular endothelium. Figure 2A is representative of a day 7 vessel. Figure 2B depicts the presence of mononuclear cell adhesion and the variability of adventitial infiltration along a

transverse section of pulmonary venule. In contrast to perivascular infiltration, peribronchiolar tissues remained normal at this time point.

Day 10 GVHD tissue sections exhibited a transitional phase of histopathology. Thirty percent of affected vessels within a tissue section demonstrated perivascular infiltration that duplicated Grade 1 criteria. However, 10% of affected vessels corresponded to Grade 2 (Mild acute rejection) (Yousem et al. 1990) guidelines. The remaining 60% of affected vessels were in transition between Grades 1 and 2 as represented by Figure 3. The escalation of perivascular infiltration and alveolar septal thickening to Grade 2 began in peripheral vessels with a progressive escalation into the center of each lung lobe. Alveolar septae were significantly widened due to an increased cellularity and edema. In addition, by day 10, 20% of the terminal and respiratory bronchioles within a whole lung tissue section were surrounded by mononuclear infiltrates 2-3 cells thick (data not shown).

The histopathology of day 14 GVHD tissue sections duplicated the parameters defined by Grade 2 (Mild acute rejection) criteria (Yousem et al. 1990). Perivascular mononuclear infiltrates, that were easily recognizable at low magnification, surrounded more than 75% of all affected venules and arterioles. Adventitial spaces were densely infiltrated by mononuclear cells. Degenerative endothelialitis and decreased luminal area were also components of Grade 2 affected vessels. Intra-luminal lymphocytes demonstrated a strong affinity for the deteriorating vascular endothelium. Figure 4 is representative of Grade 2 vessels. In addition, by day 14, lymphocytic infiltrates up to 5 cells thick were observed around 60% of the bronchioles within a whole lobe tissue section, and small areas of peribronchiolar infiltrates had begun to invade the mucosa.

F_1 rats injected with parental cells developed a late-stage of acute GVHD between days 15 and 21. The late-stage included a subset of animals killed within an estimated 24 hours of death that were classified as having end-stage disease. Imminent death was determined by the presence of severe physiologic parameters such as cachexia, diahrrea, skin lesions, deteriorated coat and kyphosis.

Tissue sections from late-stage animals demonstrated a histopathology that corresponded to Grade 3 (Moderate acute rejection) (Yousem et al. 1990) allograft rejection guidelines. Extensive perivascular infiltrates were no longer contained by intact, limiting plates and had extended into the alveolar septae producing an interstitial pneumonitis as represented in Figure 5A. Greater than 80% of affected vessels within a whole lobe section were classified as Grade 3. Late-stage pathology also included a distinct lymphocytic bronchiolitis that affected 75% of the terminal and respiratory bronchioles within a whole lung tissue section. Peribronchiolar mononuclear cells had begun to invade the mucosa, submucosa and bronchiolar epithelium. Figure 5B is representative of the lymphocytic bronchiolitis present during late-stage GVHD.

Tissue sections from end-stage GVHD animals exhibited extensive histopathology that corresponded to the criteria established for Grade 4 (Severe acute rejection) (Yousem et al 1990) rejection guidelines. Grade 4 pulmonary disease affected 70% of each tissue section, while the remaining 30% exhibited histopathology that corresponded to Grades 1 through 3 of lung allograft rejection. The presence of Grade 4 histopathology was independent of individual lung lobes. Diffuse perivascular, interstitial and peribronchiolar infiltrates of mononuclear cells were characteristic of Grade 4 disease, and permeated whole lobe tissue sections. Endothelialitis was severe and all tissues were edematous. Parenchymal tissues were

hemorrhagic with an increased number of alveolar macrophages. Densely stained pyknotic nuclei were observed within lymphatic vessels, parenchymal tissues, and peribronchiolar infiltrates. By the end-stage of acute pulmonary GVHD, lymphocytic bronchiolitis had advanced to include the larger airways resulting in lymphocytic bronchitis. Figure 6 is representative of Grade 4 histopathology during end-stage acute GVHD.

Interstitial Width and Volume Density Measurements

Figures 7A and 7B represent data obtained from the quantification of variations in alveolar septal width and perivascular infiltrate volume density respectively. Alveolar septal width and perivascular infiltrate volume density measurements were significantly greater ($p \leq 0.05$) than control values by day 7. Both measured parameters reached a significance level of $p \leq 0.001$ compared to control values by day 10, and remained at that level of significance through day 21. Overall, alveolar septal width increased 2.4 times and perivascular infiltrate volume density increased 2.6 times as compared to control values by day 21. Similarities between the onset of statistical significance, the level of significance, and the overall trends of both graphs suggested that a similar mechanism(s) was present at the capillary and vessel level throughout acute GVHD. This mechanism(s) appeared to induce the quantifiable, concurrent increase in cellularity observed within alveolar septal and perivascular adventitial compartments.

Discussion

This study was designed to examine the pulmonary histopathology of acute lethal GVHD in the adult (DA×LEW) F_1 hybrid rat without the influence of irradiation, chemotherapy, immunosuppressive drugs or overt infection. The results describe the initiation, progression and components of a sequential pulmonary pathology that occurred as a direct result of acute

GVHD, and duplicated the histopathology of lung allograft rejection (Yousem et al. 1990).

A critical aspect of this study was the exclusion of irradiation prior to the induction of acute lethal GVHD. Most studies have incorporated irradiation into experimental GVHD protocols, or compiled data from irradiated BMT patients. However, both total-body irradiation and partial upper-body irradiation are known to cause immediate alveolar epithelial damage as well as capillary endothelial damage in the absence of disease (Gross 1977, Van Den Brenk 1971). Irradiation also initiates the development of radiation pneumonitis, and exacerbates the pulmonary histopathology of GVHD (Down et al. 1992, Lehnert, Rybka and Seemayer 1986, Meyers, Flournoy and Thomas 1982). The early histopathology of irradiation damage is often overlooked during the examination of tissue by light or electron microscopy because initial alveolar epithelial and capillary endothelial changes are not widespread (Phillips 1966). In addition, radiation pneumonitis has been diagnosed routinely by pulmonary function assays that can not assess the cell damage or depopulation which initiate the latent period that precedes radiation pneumonitis (Van Den Brenk 1971).

In contrast to previous murine (Piguet et al. 1989) and human (Atkinson et al. 1991) studies, the present nonirradiated model of acute lethal GVHD produced sequential pulmonary pathology between days 7 and 21 without similar epithelial or endothelial damage. Alveolar epithelium appeared normal throughout our study as suggested by the absence of hyaline membrane disease and a lack of mitotic figures within alveolar septae.

Obvious capillary endothelial damage was also absent. Unlike a previous study (Piguet et al. 1989), we did not conduct electron microscopic studies to examine the presence of endothelial blebbing and basement membrane separation, but it is unlikely that the

nonirradiated capillary endothelium was damaged when more manifest signs of endothelial alterations, such as intra-alveolar hemorrhage and an increased number of intra-alveolar macrophages, were not observed. Ninety-eight percent of our tissue remained free from intra-alveolar hemorrhage, high levels of intra-alveolar macrophages and debris throughout the course of acute GVHD. However, hemorrhage was widespread within alveolar septae during the late-stage of disease. Furthermore, alveolar capillaries were not filled with PMN's, alveolar septae did not contain eosinophils, and alveolar septal edema was not present until after day 10.

In the present nonirradiated model, alveolar septal and perivascular infiltrates began simultaneously but remained discrete until the late-stage of GVHD. Subsequently, dense perivascular infiltrates dispersed from adjacent adventitial limiting plates and coalesced with alveolar septal infiltrates to produce interstitial pneumonitis. In addition, peribronchiolar infiltrates developed while dense perivascular infiltrates were contained within intact adventitial limiting plates.

We believe that the affinity/adherence of intra-vascular mononuclear cells to the vascular endothelium marks the onset of perivascular infiltration as a direct result of acute GVHD, and is critical to the eventual development of interstitial pneumonitis. We hypothesize that the affinity/adherence of mononuclear cells to the vascular endothelium is induced by the presence of activated intra-vascular host or donor cells. These activated cells may produce increased levels of cytokines which upgrade endothelial adhesion molecules such as ICAM-1. Cytokines such as TNF-α, IL-4 and IFN-γ have been implicated in the upregulation of adhesion molecules (Thornhill et al. 1991, Huchet et al. 1993) and the development of GVHD (Piguet et al. 1989, Clancy, Goral and Kovacs 1990). Furthermore, the upregulation of adhesion

molecules, such as ICAM-1, coincide with the mononuclear cell infiltration of other GVHD affected organs (Dustin et al. 1988, Norton et al. 1991, Norton and Sloane 1991). In addition, the reduced flow rate of activated intra-vascular mononuclear cells within small peripheral pulmonary vessels may optimize the ability of initial cytokine levels to upregulate endothelial adhesion molecules. Higher levels of cytokines produced throughout the progression of acute GVHD may increase the density of endothelial adhesion molecules within successively larger vessels and subsequently recruit larger vessels to exhibit intra-vascular mononuclear cell affinity/adherence and infiltration. In our model, perivascular infiltration began around peripheral venules and arterioles. Larger vessels were not affected until later in the disease process. Therefore, larger vessels may have been affected earlier in a previous study (Piguet et al. 1989) due to the exacerbating effects of irradiation on GVHD (Down et al. 1992, Lehnert, Rybka and Seemayer 1986).

Previous murine (Piguet et al. 1989) and human (Atkinson et al. 1991) investigations attempted to alleviate the pulmonary histopathology of acute, lethal GVHD using anti-cytokine administration and immunosuppressive therapy, respectively. Anti-TNF-α reduced only alveolar hemorrhage, while methylprednisolone and cyclosporine protocols reduced only peribronchial, perivascular and alveolar infiltrates. Neither method was able to abrogate irradiation-induced pathologies. These results reiterate our belief that the pulmonary consequences of GVHD can not accurately be assessed in an irradiated model.

The present model of acute GVHD has previously been used to document cellular changes within other organs. A significant increase in T cells occurred within spleen and lymph nodes during the first 7 days after GVHD induction (Clancy and Mauser 1981). Subsequent to the increase, T cell populations decreased significantly from day 7 to day 21 (Clancy and

Mauser 1981). Middle and later stages of this disease model also produced a significant infiltration of lymphoid cells into the periductal connective tissue of submandibular glands (Clancy, Klein and Weddle 1981) and periportal areas of the liver (Klein, Clancy and Stuart 1982). Further studies are required to determine whether the infiltration of mononuclear cells observed within the lung, submandibular gland and liver represents specific donor anti-host interactions as a result of parental lymphoid administration.

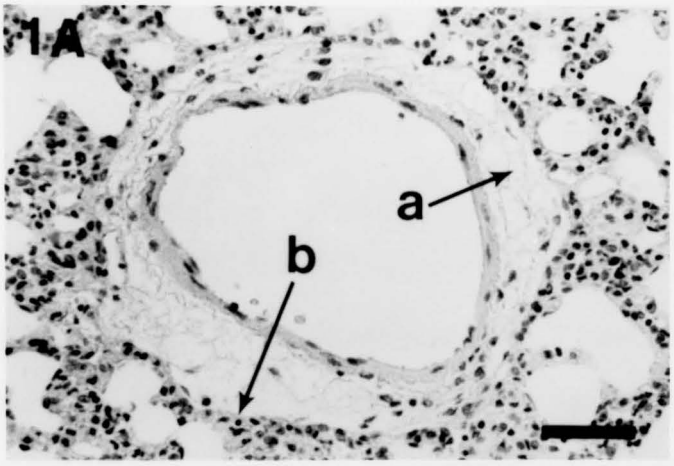
The simultaneous development of pulmonary histopathologies due to irradiation and GVHD within acute GVHD studies incorporating irradiation, prompted us to compare our observations with those that occur during lung allograft rejection. The lung allograft rejection criteria established by Yousem et al. (Yousem et al. 1990) are the most comprehensive rejection guidelines available. According to Yousem et al. (Yousem et al. 1990), acute rejection is characterized by the presence of perivascular mononuclear infiltration and lymphocytic bronchiolitis/bronchitis. Data obtained from using the present model of acute GVHD corresponded to this grading system so well that we used the guidelines of Yousem et al. (Yousem et al. 1990) to assess our histologic results. The only apparent differences between our results and the allograft rejection criteria were a lack of eosinophils and fewer neutrophils within the mononuclear infiltrates of acute GVHD. These variations may be a result of comparing human to rat tissues, or may reflect the subtle immunological differences between lung allograft rejection and GVHD pathology.

The detection of TNF-α within lung allografts (DeMeester et al. 1993) and GVHD lungs (Piguet et al. 1989, Clancy, Goral and Kovacs 1990) underscores the similarities between these transplant models. In a recent study, TNF antiserum attenuates the loss of alveolar architecture, alveolar hemorrhage, mononuclear and PMN infiltration, and intra-alveolar

proteinaceous exudates of lung allografts (DeMeester et al. 1993). However, the histopathologic equivalent to the pathogenesis present in our day 10 animals (Grades 1-2) remains. The inability of TNF antiserum to fully abrogate pulmonary pathology during lung allograft rejection and GVHD suggests that additional pathogenic mechanisms may be present in both transplant models. Furthermore, the strong correlation between the histopathology observed during lung allograft rejection and GVHD implies that similar mechanisms may be involved.

The histological and quantitative evidence obtained from this study have shown that a pulmonary syndrome characterized by the sequential development of interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis occurred as a direct result of acute GVHD. Furthermore, the histopathology produced throughout acute GVHD duplicated the pathogenesis of lung allograft rejection (Yousem et al. 1990). These findings strongly suggest that the lung is a target organ of acute lethal GVHD. Further studies will be necessary to elucidate the cellular components and mechanism(s) of pulmonary pathology throughout acute GVHD, and determine the reason why acute GVHD induces significant morphologic correlations to allograft rejection.

FIGURE 1. Photomicrographs representative of Control (Grade 0) lung tissue. (A) Adventitial space (a) and adventitial limiting plate (b) of a pulmonary venule. (B) Lung parenchyma containing alveolar septae suitable for quantification by Image Analysis (a). (H&E); Horizontal bars indicate 50 μm scale.


1A
a
b

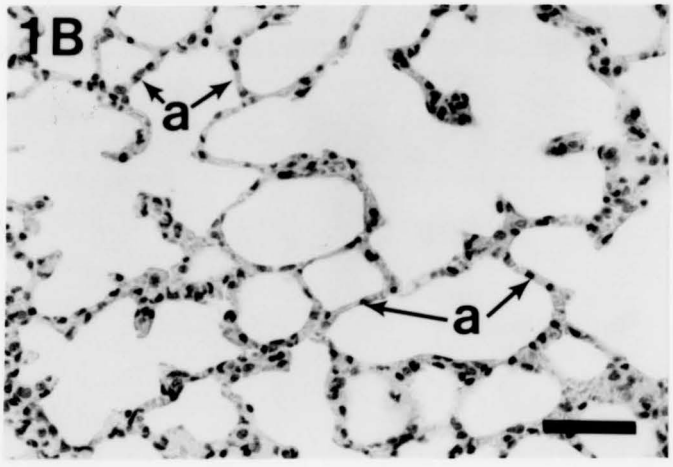
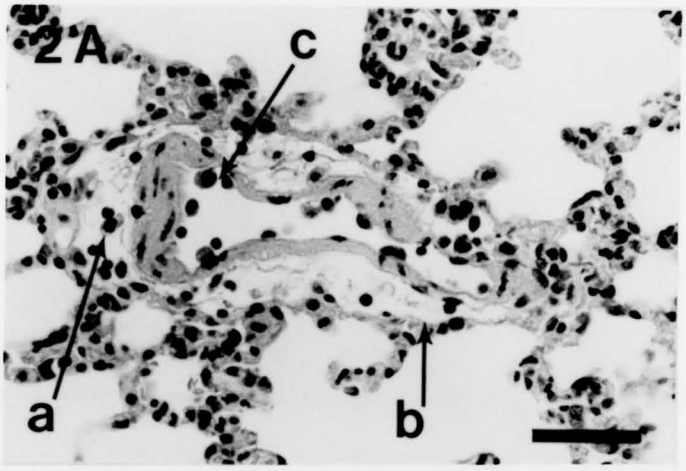

1B
a
a

FIGURE 2. Photomicrographs representative of Day 7 GVHD (Grade 1) lung tissue. (A) Venular adventitial space with early perivascular infiltrate (a), intact limiting plate (b), and intraluminal mononuclear cell adherence (c). (B) Transverse section of a venule with perivascular infiltrate (a) and intraluminal mononuclear cell adherence (b). (H&E); Horizontal bars indicate 50 μm scale.


2 A
c
a
b

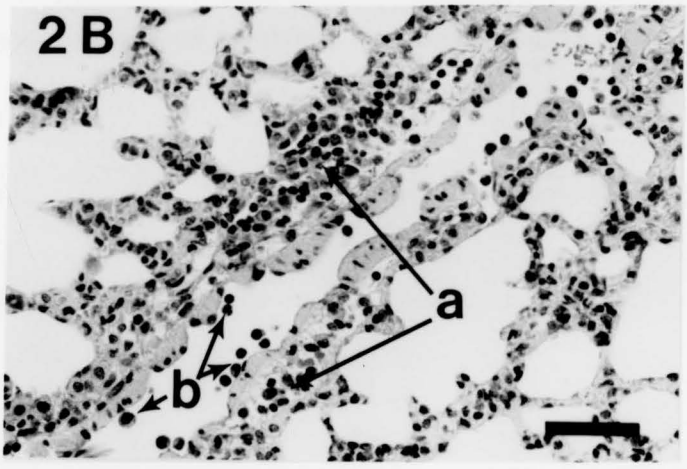

2 B
a
b

FIGURE 3. Photomicrograph representative of Day 10 GVHD (Grades 1-2) lung tissue. Increased perivascular infiltrate (a), intact limiting plate (b), and alveolar septa suitable for quantification by Image Analysis (c). (H&E); Horizontal bar indicates 50 μm scale.

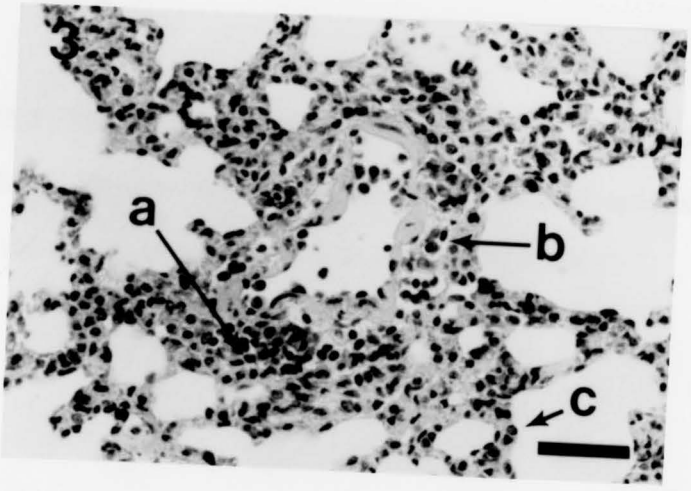

a
b
c

FIGURE 4. Photomicrograph representative of Day 14 GVHD (Grade 2) lung tissue. Perivascular infiltrate (a), degenerative endothelialitis (b), decreased vascular lumen (c), and intact limiting plate (d). (H&E); Horizontal bar indicates 50 μm scale.

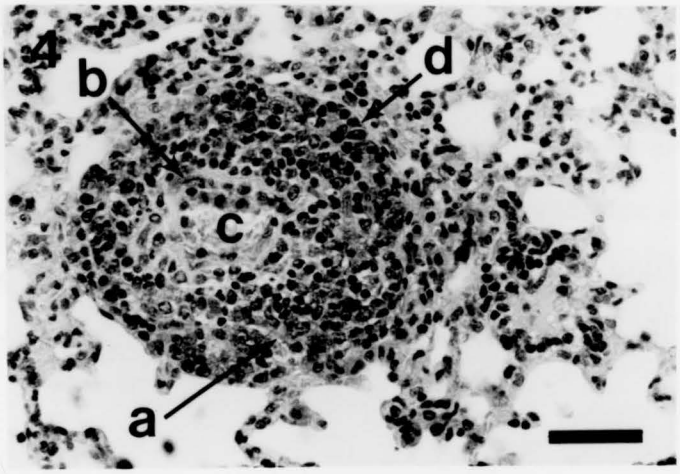

b
d
c
a

FIGURE 5. Photomicrographs representative of Late-stage GVHD (Grade 3) lung tissue. (A) Perivascular infiltrates extending into adjacent alveolar septae producing interstitial pneumonitis (a). (B) Mucosal (a), submucosal (b) and epithelial infiltration of peribronchiolar tissue by mononuclear cells producing a lymphocytic bronchiolitis (c). (H&E); Horizontal bars indicate 50 μm scale.

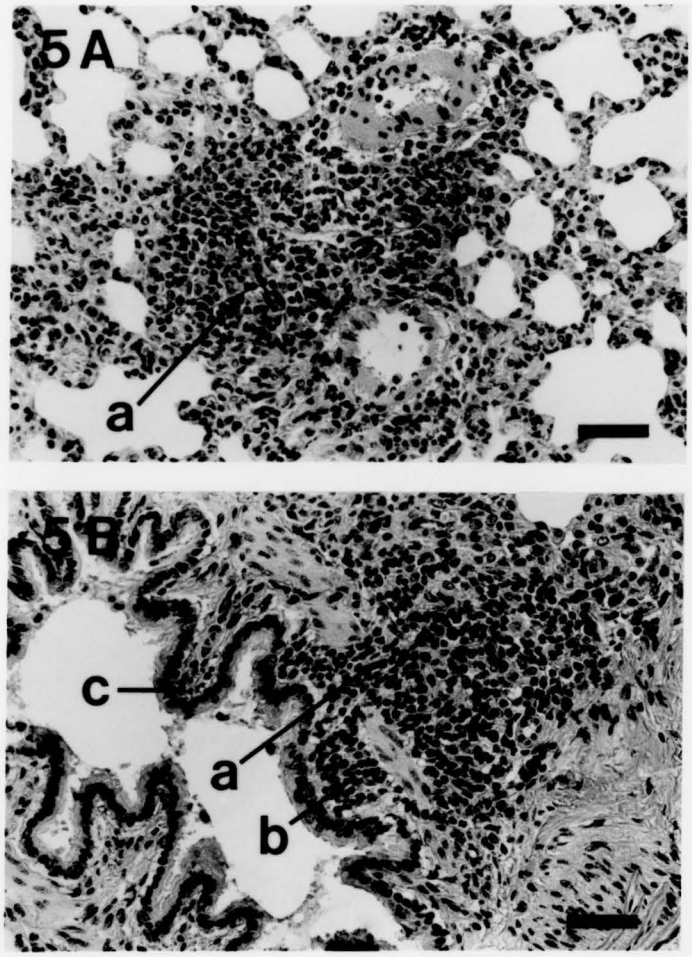

5A
a
5B
c
a
b

FIGURE 6. Photomicrograph representative of End-stage GVHD (Grade 4) lung tissue. Prominent interstitial pneumonitis (a). (H&E); Horizontal bar indicates 50 μm scale.

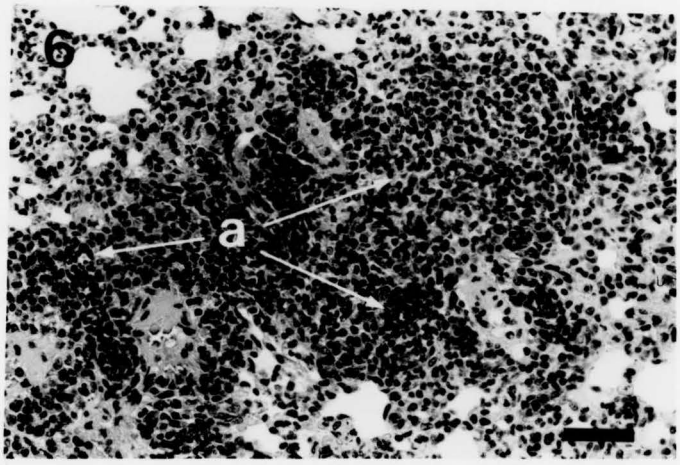

6
a

FIGURE 7. Alveolar septal width and perivascular infiltrate volume density in noninjected (DA × LEW) F_1 controls (day 0), and DA/F_1 GVHD-induced, nonirradiated adult rats 3, 7, 10, 14, and 15-21 days following injection. Data were obtained using quantitative Image Analysis. Error bars represent means ± SEM. Statistical analyses were conducted using a one-way ANOVA and Tukey post-hoc testing. (▪) = $p \leq 0.05$ and (▪▪) = $p \leq 0.001$ as compared to control values. (A) N = 6 animals per time point and 750 alveolar septal width measurements per animal. (B) N = 6 animals per time point and 500 perivascular infiltrate volume density measurements per animal.

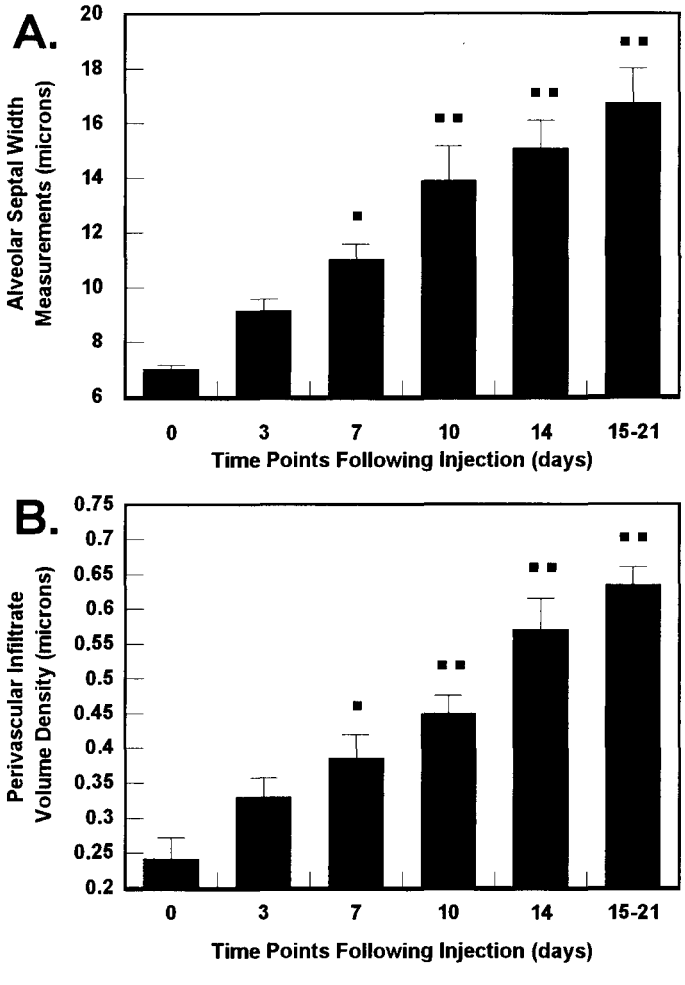

A.
Alveolar Septal Width Measurements (microns)
20
18
16
14
12
10
8
6
0
3
7
10
14
15-21
Time Points Following Injection (days)
B.
Perivascular Infiltrate Volume Density (microns)
0.75
0.7
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0
3
7
10
14
15-21
Time Points Following Injection (days)

Figure 7

CHAPTER V

PHENOTYPIC ANALYSIS OF PULMONARY PERIVASCULAR MONONUCLEAR INFILTRATES THAT OCCUR AS A DIRECT RESULT OF ACUTE LETHAL GRAFT-VS.-HOST DISEASE DESCRIBES THE ONSET OF INTERSTITIAL PNEUMONITIS

Abstract

We recently determined that the sequential development of interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis occurs as a direct result of acute lethal GVHD. Interstitial pneumonitis develops prior to lymphocytic bronchiolitis/bronchitis primarily from the dissemination of perivascular mononuclear infiltrates. We have used the adult, nonirradiated (DA × LEW) F_1 hybrid rat in the absence of chemotherapy, immunosuppression, or overt infection to determine the phenotype of infiltrating perivascular mononuclear cells throughout acute lethal GVHD. F_1 animals were intravenously injected with 1×10^6 DA parental lymphoid cells/g body weight which produced 100% morbidity and mortality by day 21. GVHD animals were killed on days 3, 7, 10, 14, and 15 - 21 following injection. Whole left lungs were frozen, serially sectioned (4μm) and incubated with a panel of mouse anti-rat monoclonal antibodies. Labeled antibody density was determined by computerized Image analysis. A biphasic pattern of infiltration was observed first for $ED1^+$, $OX8^+$ and $W3/25^+$ cells then $OX41^+$, $W3/13^+$ and $OX19/52^+$ populations. OX6 was expressed in control tissues and all time points. $OX12^+$, $OX39^+$ and $MOM/3F12/F2^+$ cells were not quantifiable. These class II^+, T-cell and macrophage populations initiated interstitial pneumonitis and indirectly pointed to an activated T-cell, cytokine-induced upregulation of adhesion molecules that may facilitate transportation of CMV.

Introduction

Graft-vs.-Host Disease (GVHD), a frequent and often lethal complication of allogeneic bone marrow transplantation (BMT), develops when donor T lymphocytes initiate an immune response to disparate host major and minor histocompatibility antigens (Korngold and Sprent 1990). However, depletion of T lymphocytes from the donor inoculum often abrogates engraftment and increases the rate of leukemic relapse (Korngold and Sprent 1990, Tutschka et al. 1987, Weiden et al. 1981). The acute form of GVHD incurs a high incidence of mortality and diagnosis remains difficult due to the complicating effects of pre-transplant conditioning regimens. Therefore, defining the immunopathologic consequences of acute GVHD remains fundamental to the clinical management of allogeneic BMT patients.

Pulmonary complications are a significant cause of mortality during acute GVHD. Many studies have implicated interstitial pneumonitis and lymphocytic bronchitis as the prevalent histopathologic elements of acute GVHD (Atkinson et al. 1991, Sloane and Norton 1993, Sloane et al. 1983, Beschorner et al. 1978, Neiman et al. 1977, Sullivan et al. 1986). However, these studies have not determined a correlation between the onset of interstitial pneumonitis and lymphocytic brochiolitis/bronchitis or the development of GVHD-induced pulmonary symptoms. In a recent study, we determined that a pulmonary syndrome characterized by the sequential development of interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis occurs as a direct result of acute lethal GVHD (Workman and Clancy 1994). In the absence of irradiation, chemotherapeutic agents, immunosuppressive drugs or overt infection, we demonstrated that acute GVHD-induced interstitial pneumonitis develops prior to lymphocytic bronchiolitis/bronchitis through the coalescence of perivascular and alveolar septal mononuclear cell infiltrates. In addition, the histopathology produced through the development of interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis duplicates the

pathogenesis of lung allograft rejection. Consequently, our previous study established that the lung is an important target organ of acute lethal GVHD.

Perivascular mononuclear cell infiltration appears to be the most prominent, quantifiable and reliable parameter of developing acute pulmonary GVHD (Workman and Clancy 1994). Consequently, in the present study we have used the adult, nonirradiated (DA × LEW) F_1 hybrid rat and a comprehensive panel of mouse anti-rat monoclonal antibodies to: (1) determine the phenotype of perivascular mononuclear cell infiltrates throughout the course of acute GVHD, (2) assess the "position" of labeled mononuclear cells within the perivascular adventitial space relative to the adventitial limiting plate (Workman and Clancy 1994, Yousem et al. 1992), and (3) quantify the density of antibody-labeled perivascular mononuclear cell populations throughout the course of acute lethal GVHD.

Materials and Methods

Animals

Lewis (LEW:RT1^l), DA (RT1^a) and (DA×LEW) F_1 hybrid rats were bred in the animal care facility at Loyola University, Stritch School of Medicine. Acute, lethal GVHD was induced in 8 - 12 week old nonirradiated (DA×LEW) F_1 rats by the intravenous injection of 1×10^6 DA lymphoid (spleen and lymph node) cells/gram body weight as previously described (Clancy and Mauser 1981). All F_1 animals injected with parental DA lymphoid cells developed classic signs of acute systemic GVHD by day 14, and 100% morbidity as well as mortality by day 21.

To establish a control group, the lungs were excised on days 3 and 7 from 4 (2 animals per time point) noninjected F_1 rats, 4 GVHD-induced F_1 rats, and 4 F_1 animals intravenously injected with 1×10^6 F_1 lymphoid cells/gram body weight. All F_1 animals were

matched to litter and sex. Histological and quantitative data confirmed that there were no differences between noninjected and syngeneically-injected F_1 animals compared on days 3 and 7. In addition, the pulmonary histopathology present by day 7 in GVHD-induced F_1 animals was absent in both noninjected and syngeneically-injected F_1 groups. Therefore, noninjected adult, nonirradiated F_1 hybrid rats were used as controls throughout this study.

Noninjected F_1 control and GVHD-induced animals were killed by ether overdose. GVHD animals were killed on days 3, 7, 10, 14, and 15 - 21 (5 animals per time point) following injection. GVHD animals were matched to each other and corresponding controls by litter and sex when possible. All animal manipulations were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago.

Immunohistochemistry

The heart, lungs and trachea were removed intact, and all four right lung lobes were prepared for histological analysis as previously described (Workman and Clancy 1994). A 1 mm segment of distal left lung was removed to allow for drainage of fixation materials. The left lung was intra-tracheally injected with 50 cc of 4% paraformaldehyde (PFA) chilled to 4^0 C until the lung lobe was completely distended for at least 30 seconds. After PFA injection, 20 cc of Ames OCT Compound (Miles Lab, Inc., Elkhart, Ind.) was intra-tracheally injected until the OCT flowed from the distal lobe incision. The left lung was dissected from the left mainstem bronchi and divided in half horizontally. Each lung half was placed in 4% PFA for 2 - 4 hours at 4^0 C. After PFA submersion, each lung half was placed in 20% sucrose at 4^0 C for 12 - 48 hours. Following sucrose immersion, all tissues were submersed in OCT compound, frozen in liquid nitrogen and stored at -70^0 C. Whole spleens (positive control) and kidneys (negative control) were also dissected, fixed and frozen according to the above immunohistochemical

protocol.

Serial sections (4 μm) were cut from each lung, spleen and kidney tissue specimen along an anterior-posterior frontal plane on a Leitz 1720 Digital Cryostat at -20^0 C. Sections were arranged two per slide providing serial sections on successive slides that enabled immunostaining of separate antibodies on serial sections. Each slide contained two whole lobe lung, kidney or spleen serial sections from differing tissue layers. The slides were mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA), air-dried and stored at -20^0 C.

Immunohistochemical analysis was conducted using an avidin-biotin staining method. All tissue sections were initially blocked with 10% fetal calf serum (FCS) in 0.1 M Tris buffer (pH 7.2) for 30 minutes at 22^0. Each primary mouse anti-rat monoclonal antibody (Bioproducts for Science, Inc., Serotec, Indianapolis, In) (Table 20) was individually titrated on sections of lung, spleen (positive control) and kidney (negative control) to achieve optimal dilutions in 10% FCS in Tris buffer and incubated for 1 hour at 22^0. Sections were subsequently washed in Tris buffer with 1% Triton-X (2 × 10 min) followed by Tris buffer without Triton-X (2 × 10 min). The secondary antibody, biotinylated sheep anti-mouse Ig (1:200) (Amersham, Arlington Hts, IL), was diluted in 10% FCS in Tris buffer and incubated 1 hour at 22^0. Endogenous peroxidase was blocked by treatment with hydrogen peroxide (0.3%) in 100% methanol (30 min). Standard peroxidase-conjugated streptavidin (1:200) (Dako, Carpinteria, CA) and DAB (Sigma Chemical Co, St. Louis, Mo) protocols were used to visualize the chromagen. All DAB-stained tissues were intensified with Osmium Tetroxide (Electron Microscopy Sciences, Fort Washington, PA), dehydrated, counter-stained with hematoxylin, and mounted in Permount. Controls consisted of adjacent sections incubated either with normal mouse serum (matched for protein concentrations) or isotype-matched antibodies (UPC10, IgG_{2a}

and MOPC 21, IgG_1) (Sigma) in place of the respective antibodies. Each of these controls was negative.

In addition to immunohistochemical staining, all tissues were tested with Brown-Brenn Gram and Grocotts Methenamine Silver stains to detect the presence of bacteria and fungi, respectively. CMV inclusion bodies were absent within all lung tissues tested according to light microscopic examination. Immunohistochemical staining for cytomegalovirus (CMV) was conducted using a combination of two monoclonal mouse anti-human antibodies, DDG9 as well as CCH2 (Dako, Carpenteria, CA) (Wirgart et al. 1990, Niedobitek et al. 1988) to confirm the absence of CMV. Mouse anti-human antibodies were used because mouse anti-rat mononclonal antibodies to CMV were not available. Tissues were randomly selected from animals killed on days 0 (control), as well as 7, 14, and 18 days following injection to rule out the presence of infection. All tissues were devoid of viral, fungal or bacterial contamination.

Image Analysis

Image analysis was accomplished using NIH Image installed on an Apple Macintosh IIcx computer interfaced to a Leitz (Optische Werke; Wetzlar, Germany) microscope using a Scion Image Capture Board (Scion Corp.; Frederick, MD) and a MOS Javelin Solid State video camera (Javelin Electronics; Torrance, CA). All images were digitized at a magnification of 40X. Prior to each session, the microscope lamp was turned on for 30 minutes to stabilize the illumination source. Uniformity of the optical system was enhanced by automatically subtracting a blank field from a part of the slide not containing tissue with the illumination adjusted to an average pixel value of 127 on the Look-Up Table (LUT). The optical density corresponding to specific tissue components was calculated on the LUT as determined by preliminary analyses of random sections (Table 20).

A grid was designed to provide random, systematically reproducible tissue sites for quantification by Image Analysis. The grid pattern was superimposed on the center of each immunohistochemically-stained lung section. Points along the grid pattern were 80 µm apart and covered the entire tissue section without producing points outside the borders of the section. If a grid site did not contain measurable tissue it was not used.

Thirty animals (5 animals per time point) were used in this study. Ten random, systematically reproducible tissue sites were measured per tissue section, using the grid and selection criteria described above. To quantitate the density of antibody-labeled mononuclear cells (volume density) within perivascular infiltrates, circumferential and area measurements were obtained for the lumen and adventitial limiting plate of selected pulmonary vessels. The final volume density of the adventitial space was based on the optical density of perivascular infiltration as calculated by the LUT values described above.

Statistics

Mean values of the measurements obtained for perivascular infiltrate volume density were calculated for each tissue section. The final means from each animal were grouped according to the time point tested. Differences between the overall, cumulative means derived from each time point were tested for statistical significance. Each antibody-labeled cell population was assessed for statistical significance by comparing the volume density measurements obtained from the first time point that yielded quantifiable results to subsequent time points. One-way Analysis of Variance and Tukey post-hoc analysis (Spatz and Johnson 1984) were used to provide continuity with standards employed to assess the histopathology of acute pulmonary GVHD as previously described (Workman and Clancy 1994).

Results

Volume Density

Of the ten monoclonal antibodies selected for immunohistochemical analysis, seven provided staining within whole lobe lung tissues (Table 20). However, OX12 (Mason et al. 1983), OX39 (Paterson et al. 1987) and MOM/3F12/F2 (Billett, Gunn and Mayer 1984) antibodies did not exhibit staining within the perivascular adventitial space at any time point tested. Peak volume density measurements for OX6-, ED1-, OX8-, W3/13-, W3/25- and OX19/52-labeled mononuclear cells were obtained on day 14. Only volume density measurements of OX41-labeled mononuclear cells were maximal between days 15 - 21. The slight drop in volume density measurements within OX6-, OX8- and W3/25-labeled mononuclear cell populations between days 15-21 resulted from dissolution of the perivascular adventitial limiting plate and a subsequent increase within the total area measured. Decreased volume density measurements from ED1-, W3/13- and OX19/52-labeled mononuclear cell populations resulted from both a decrease in labeled perivascular cells and dissolution of the perivascular adventitial limiting plate.

OX6

Figure 8 A is representative of OX6 (McMaster and Williams 1979, Mason, Dallman and Barclay 1981) staining within the perivascular adventitial spaces of control tissues. One to three OX6-labeled mononuclear cells were found consistently within the narrow perivascular space. The small perivascular volumes combined with the presence of OX6-labeled cells produced exaggerated volume density values for day 0 (control) (Figure 10). Day 3 volume density measurements were substantially reduced from control values and became more representative of histological data as previously described (Workman and Clancy 1994). Day 3 volume density measurements were reduced because the number of OX6-labeled mononuclear

cells within day 3 perivascular spaces remained consistent with the number of labeled cells in the perivascular spaces of control tissues, yet the perivascular spaces in day 3 tissues were increased. As a consequence of this finding, OX6 data from days 7, 10, 14 and 15 - 21 were statistically compared to day 3 volume density measurements. Day 10 OX6 volume density measurements increased 2.8 times ($P \leq 0.05$) as compared to day 3 values. Days 14 and 15-21 increased 3.7 - 3.6 times respectively ($P \leq 0.01$) as compared to day 3 measurements (Figure 10).

Figure 8 B is representative of OX6 labeling obtained in the advanced stages of day 14 perivascular mononuclear cell infiltration. During initial perivascular infiltration on day 7, OX6-labeled mononuclear cells were adjacent to the perivascular limiting plate (data not shown). By day 14, OX6-labeled cells appeared to be uniformly distributed within the perivascular space (Figure 8 B).

ED1

Figure 8 C is representative of ED1(Dijkstra et al. 1985) labeling in the early stages of day 14 perivascular mononuclear cell infiltration. Day 14 volume density measurements were increased 2.4 times ($P \leq 0.01$) as compared to day 7 values (Figure 11 A). Volume density measurements obtained between days 15 - 21 returned to the same overall value calculated for ED1-labeling on day 10. During early infiltration of the perivascular space on day 7, ED1-labeled cells were positioned midway between the endothelium and perivascular limiting plate (data not shown). By day 14, ED1-labeled mononuclear cells were observed dispersed throughout the perivascular space (Figure 8 C).

OX41

Figure 8 D is representative of OX41(Robinson, White and Mason 1986) labeling in

the early stages of perivascular mononuclear cell infiltration between days 15 - 21. Although volume density measurements from days 15 - 21 increased 1.7 times over day 14 values, the increase was not calculated to be significant (Figure 11 B). However, OX41 was the only monoclonal antibody used in the present study that showed increased labeling between days 15 - 21. During early infiltration of the perivascular space on day 14, OX41-labeled mononuclear cells were adjacent to the adventitial limiting plate (data not shown). Between days 15 - 21, OX41-labeled mononuclear cells were dispersed throughout the adventitial space (Figure 8 D).

OX8

Figure 9 A is representative of OX8 (Mason et al. 1983, Barclay 1981, Dallman, Mason and Webb 1982) staining in the early stages of day 14 perivascular mononuclear cell infiltration. Although day 14 volume density measurements increased 1.8 times over day 7 values, the increase was not calculated to be significant (Figure 12 A). During early infiltration of the perivascular space on day 7, OX8-labeled mononuclear cells were located within the perivascular adventitial space midway between the endothelium and adventitial limiting plate (data not shown). By days 15 - 21, OX8 labeled mononuclear cells were dispersed throughout the perivascular space (Figure 9 A).

W3/13

Figure 9 B is representative of W3/13 (Brown et al. 1981, Dyer and Hunt 1981) staining within the perivascular mononuclear cell infiltration characteristic of day 14. Although day 14 volume density measurements increased 2.0 times over values obtained between days 15 - 21, the increase was not calculated to be significant (Figure 12 B). During initial infiltration on day 14, W3/13-labeled mononuclear cells were positioned midway between the endothelium

and adventitial limiting plate. Although volume density measurements from days 15 - 21 were decreased, W3/13-labeled cells were dispersed throughout the perivascular space (Figure 9 B).

W3/25

Figure 9 C is representative of W3/25 (Mason et al. 1983, Barclay 1981, Brideau et al. 1980) staining within the perivascular mononuclear cell infiltration characteristic of day 14. Days 14 and 15 - 21 were increased 3.8 - 3.4 times respectively ($P \leq 0.01$) over day 7 volume density measurements (Figure 13 A). During the early infiltration of mononuclear cells on day 7, W3/25-labeled cells were observed midway between the endothelium and adventitial limiting plate (data not shown). By day 14 W3/25-labeled cells were dispersed throughout the perivascular space (Figure 9 C).

OX19/52

OX52 (Robinson, Puklavec and Mason 1986) has been shown to augment OX19 (Dallman, Thomas and Green 1984) staining on cryostat sections (Robinson, Puklavec and Mason 1986). Preliminary data for the present study also determined that neither OX19 nor OX52 alone exhibited the intensity of immunohistochemical staining that was obtained when both antibodies were combined. Consequently, an equal mixture of OX19 and OX52 was used to indicate pan T-cell phenotyping throughout this study.

Figure 9 D is representative of OX19/52 staining in the early stages of day 14 perivascular mononuclear cell infiltration. Although day 14 volume density measurements increased 2.2 times over values obtained between days 15 - 21, the increase was not calculated to be significant (Figure 13 B). During early infiltration of mononuclear cells on day 14, OX19/52-labeled cells were positioned midway between the endothelium and adventitial

limiting plate. Although volume density measurements from days 15 - 21 were decreased, OX19/52-labeled cells were dispersed throughout the perivascular space (Figure 9 D).

Summary

Immunohistochemical analysis and subsequent computerized image analysis of antibody-labeled pulmonary perivascular mononuclear cell infiltrates quantified volume density fluctuations within 7 out of 10 selected mouse anti-rat monoclonal antibodies. Volume density measurements indicated a biphasic class II $^{+}$, T cell and macrophage mediated response. OX6-, OX8-, ED1- and W3/25-labeled mononuclear cells appeared to be adherent to the vascular endothelium and were detected during initial perivascular infiltration by day 7. Whereas, OX41-, W3/13- and OX19/52-labeled mononuclear cells appeared to be adherent to the vascular endothelium on day 10, but were not observed within the perivascular space until day 14.

Discussion

The present study is the first to determine a phenotypic profile of mononuclear cells present within the pulmonary perivascular infiltrates that characterize interstitial pneumonitis and occur as a direct result of acute GVHD. We employed immunohistochemical analysis to define the phenotypic components of pulmonary perivascular infiltrates within the adult (DA × LEW) F_1 hybrid rat in the absence of chemotherapy, irradiation, immunosuppressive drugs or overt infection. Our data determined that the process of perivascular infiltration was produced through a biphasic influx of OX6^{+}, T-cell and macrophage populations. These results have also provided indirect evidence that the early infiltration of T-cell subsets may facilitate a route for CMV infection.

The primary target organs of acute GVHD, such as spleen, lymph nodes, skin, G.I. tract, and liver exhibit increases in both MHC class I and class II antigen expression (Korngold and Sprent 1990, Lampert et al. 1982, Lever et al. 1986, Breathnach and Katz 1983, McDonald 1988). As a result of MHC expression, these GVHD-target organs become the primary sites of T-cell infiltration. Class II expression is routinely found on cells within the perivascular and subpleural spaces of normal murine lung (Skoskiewicz et al. 1985), but does not appear on normal rat epithelial or endothelial cells (Romaniuk et al. 1987). In our present study, the concurrent perivascular infiltration of cytotoxic and helper T-cell subsets appeared to correlate with the presence of MHC class I and class II expression. Furthermore, OX6-labeled mononuclear cells within control perivascular spaces appeared to be macrophages which increased throughout acute GVHD and preceded T-cell infiltration. In contrast to perivascular labeling, OX6 did not label pulmonary endothelium at any time point following GVHD induction. A similar absence of class II expression is noted on murine vascular endothelial cells throughout rat lung allograft rejection (Romaniuk et al. 1987). The present OX6-labeled immunohistochemical data were consistent with this previous study and corroborated our prior conclusions that pulmonary histopathology throughout acute GVHD duplicates lung allograft rejection and characterizes the lung as an important target organ following allogeneic BMT (Workman and Clancy 1994).

ED1 and OX41 were used to assess the presence of macrophage subsets within perivascular infiltrates. Both $ED1^+$ and $OX41^+$ cells appeared to display a preferential affinity for the adventitial limiting plate which is composed primarily of Type IV collagen (Yousem et al. 1992). During lung allograft rejection, Type IV collagenase staining is observed on perivascular mononuclear cells adjacent to the limiting plate (Yousem et al. 1992). There was a striking coincidence between the increased volume density of OX41-labeled cells between days 15 - 21,

the position of OX41-labeled cells adjacent to the limiting plate, and subsequent dissolution of the limiting plate to produce interstitial pneumonitis. It is possible that ED1-labeled cells may release cytokines or collagenase to weaken the extracellular matrix and limiting plate, while OX41-labeled mononuclear cells may produce the type IV collagenase required to initiate dissolution. Further study is needed to determine why the volume density measurements of ED1-labeled cells obtained between days 15 - 21 dropped to day 10 values while volume density measurements for OX41-labeled mononuclear cells increased 2-fold over day 14 values. The presence of OX41-labeled cells during peak volume density values for ED1-labeled cells suggested that two different macrophage populations were being labeled by ED1 and OX41 antibodies.

OX8, W3/13, W3/25 and OX19/52 antibodies were used to assess the presence of T-cell subsets within perivascular infiltrates. $OX8^+$ and $W3/25^+$ cells were both observed within perivascular infiltrates on day 7. Volume density measurements exhibited similar rates of increase within OX8 and W3/25 antibody-labeled populations between days 7 and 10, as well as similar rates of volume density decrease between days 14 and 15 - 21. However, OX8 volume density measurements on days 7 and 10 comprised a higher portion of overall OX8-staining than W3/25 volume density measurements for the same time points. W3/25 volume density measurements produce a similar staining trend to ED1-labeled cells except for high values between days 15 - 21. High volume density values for W3/25-labeled cells between days 15 - 21 may indicate that a different epitope of OX41-labeled macrophages was incorporated into W3/25 labeling.

Both W3/13 and OX19/52 antibodies labeled intravascular mononuclear cells on day 10 but neither antibody was observed within perivascular infiltrates until day 14. Similar rates of

increase and decrease occurred for volume density measurements obtained from W3/13- as well as OX19/52-labeled perivascular mononuclear cell populations on days 14 and 15 - 21. Relative to the volume density measurements within each population, both W3/13 and OX19/52 values were reduced by half between days 15 - 21. Although OX19/52-labeling provided appropriate pan T-cell staining within spleen tissues, it did not represent the total of perivascular T-cell subset labeling in acute GVHD lungs. We believe that either the OX19 and OX52 receptors may be altered traversing the endothelium, or the strength of OX19/52 staining may be organ specific and naturally low within the pulmonary environment.

OX41-, W3/13- and OX19/52-labeling were observed only on intravascular mononuclear cells within day 10 lung tissues. This data suggested that OX41-, W3/13- and OX19/52-labeled cell populations were not present in peripheral blood immediately following the induction of acute GVHD. Previous acute GVHD studies using this model showed similar fluctuations within the lymphoid populations of other GVHD target organs. A significant increase in W3/13-labeled T cells occurred within spleen and lymph nodes during the first 7 days after GVHD induction (Clancy 1984). Subsequently, W3/13-labeled spleen populations showed a significant decrease between days 7 and 21 while a significant increase of W3/13-labeled cells occurred within the submandibular glands and periportal areas of the liver (Clancy 1984, Klein, Clancy and Stuart 1982, Clancy, Klein and Weddle 1981). Consequently, we believe that W3/13-, OX41-, and OX19/52-labeled cells were not apparent within the perivascular space until day 14 because prior to infiltration they were localized within the lymphoid compartments of spleen and lymph nodes. In contrast, ED1-, OX8- and W3/25-labeled mononuclear cells may have remained within the circulating pool of peripheral blood where they were available for initiation of perivascular infiltration on day 7.

MOM/3F12/F2-, OX12- and OX39-labeling were not found within the perivascular space at any time point throughout acute GVHD. However, all three antibodies demonstrated appropriate labeling patterns within positive and negative control tissues as well as minimal staining within the lung parenchyma. Immunohistochemical analysis conducted with the MOM/3F12/F2 antibody confirmed the absence of neutrophils observed within perivascular infiltrates during histological examination (Workman and Clancy 1994). B cells were not present within perivascular infiltrates and none were observed after OX12-labeling. One to three OX39-labeled cells were found within each whole lobe lung section, but OX39-labeled cells were not observed within perivascular infiltrates at any time point following injection. It is possible that the affinity of OX39 for the IL-2r may be altered within the pulmonary environment for unknown reasons, or IL-2 may not play a role in the pulmonary histopathology of acute GVHD. A noticeable lack of cellular proliferation as determined by the absence of mitotic figures also suggested that IL-2 may not act locally during the pathogenesis of acute pulmonary GVHD.

The affinity/adherence of intravascular mononuclear cells to the vascular endothelium marks the onset of perivascular infiltration as a direct result of acute GVHD and is critical to the eventual development of interstitial pneumonitis. We believe that the affinity/adherence of mononuclear cells to the vascular endothelium is induced by the presence of activated intravascular donor cells. These activated cells produce increased levels of cytokines such as IFN-γ, TNF-α, IL-1 (Springer 1990, Duquesnoy, Trager and Zeevi 1991) and IL-4 that induce class II expression, upregulate endothelial adhesion molecules and augment the development of GVHD (Thornhill et al. 1991, Huchet et al. 1993, Piguet et al. 1989, Clancy, Goral and Kovacs 1990, Pober et al. 1986). Activated T_h1 cells are known to release IFN-γ which increases class II expression, TNF-α production by macrophages, and the upregulation of

ICAM-1(Duquesnoy, Trager and Zeevi 1991). TNF-α in combination with IFN-γ and IL-4 plays a principal role in the recruitment of a predominantly mononuclear infiltrate across the vascular endothelium through LFA-1/ICAM-1 mechanisms (Thornhill et al. 1991). Immunohistochemical studies on frozen sections from rejecting lung allografts reveal an intense staining pattern of TNF-α within pulmonary interstitial mononuclear cells during a period of rejection that duplicates the histopathology observed on day 14 of our GVHD model (DeMeester et al. 1993, Workman and Clancy 1994). In addition, IFN-γ and TNF-α induce upregulation of adhesion molecules such as ICAM-1 which also coincides with the mononuclear cell infiltration of other GVHD affected organs (Dustin et al. 1988, Norton et al. 1991, Norton and Sloane 1991). Previous studies have documented that antibodies to IFN-γ and TNF-α significantly abrogate the histopathology of GVHD-induced skin and intestinal lesions (Piguet et al. 1987, Mowat 1989) as well as the perivascular mononuclear infiltration of rejecting lung allografts (DeMeester et al. 1993).

In addition to the expression of LFA-1, activated T cells also express VLA adhesion molecules which mediate the interaction between lymphocytes that have crossed the endothelium and components of the extracellular matrix (Springer 1990). VLA antigens may have played a role in positioning early OX8-, W325-, W3/13- and OX19/52-labeled T cells midway between the endothelium and adventitial limiting plate, while early ED1- and OX41-labeled macrophage subsets were found adjacent to the limiting plate.

In the present study we have been careful to avoid contamination by CMV which is symptomatic in 50 - 80% of allogeneic BMT patients and incurrs a mortality rate of 80 - 90% as a result of interstitial pneumonitis (Meyers, Flournoy and Thomas 1980, Paulin et al. 1986, Neiman et al. 1977, Bortin et al. 1982). Yet, after comparing our results to the data obtained in

other acute GVHD studies, a strong correlation emerged between the $OX8^+$, $W3/25^+$ T-cell subsets integral to the development of GVHD-induced interstitial pneumonitis and the donor Lyt 2^+ and $L3T4^+$ T-cell subsets required to produce a CMV-induced pneumonitis (Shanley et al. 1987).

It is possible that the upregulation of LFA-1/ICAM-1 and VLA adhesion pathways on activated T cells which are required to produce a GVHD-induced interstitial pneumonitis may also facilitate a route of CMV infection. ICAM-1 acts as a ligand for several viruses such as rhinovirus and some strains of coxsackie virus (Greve et al. 1989, Martin et al. 1990). Although rhinoviruses have evolved into more than 100 non-crossreactive antigenic variants, 90% bind to ICAM-1(Springer 1990). LFA-1 molecules on activated T cells and rhinoviruses both bind to overlapping yet distinct regions of the first immunoglobulin-like domain of ICAM-1 (Greve et al. 1989, Martin et al. 1990, Staunton et al. 1990, Staunton et al. 1989). Therefore, we hypothesize that the release of cytokines by activated donor T cells and the subsequent upregulation of adhesion molecules may attract latent CMV particles to form a CMV/T-cell/ICAM-1 complex (studies in progress). The ensuing perivascular infiltration of CMV and activated donor T cells may explain the difficulty in distinguishing between the onset of acute GVHD and CMV pneumonitis.

The present study has shown that the perivascular infiltration characteristic of interstitial pneumonitis occurrs as a biphasic influx of $OX6^+$, $ED1^+$, $OX8^+$ and $W3/25^+$ mononuclear cells on day 7, as well as $OX41^+$, $W313^+$, and $OX19/52^+$ populations on day 14. The release of cytokines and collagenase by these perivascular mononuclear cells may help to weaken the adventitial matrix and facilitate dissolution of the perivascular limiting plate. Early immunohistochemical analysis of transbronchial biopsies may help to diagnosis the onset of

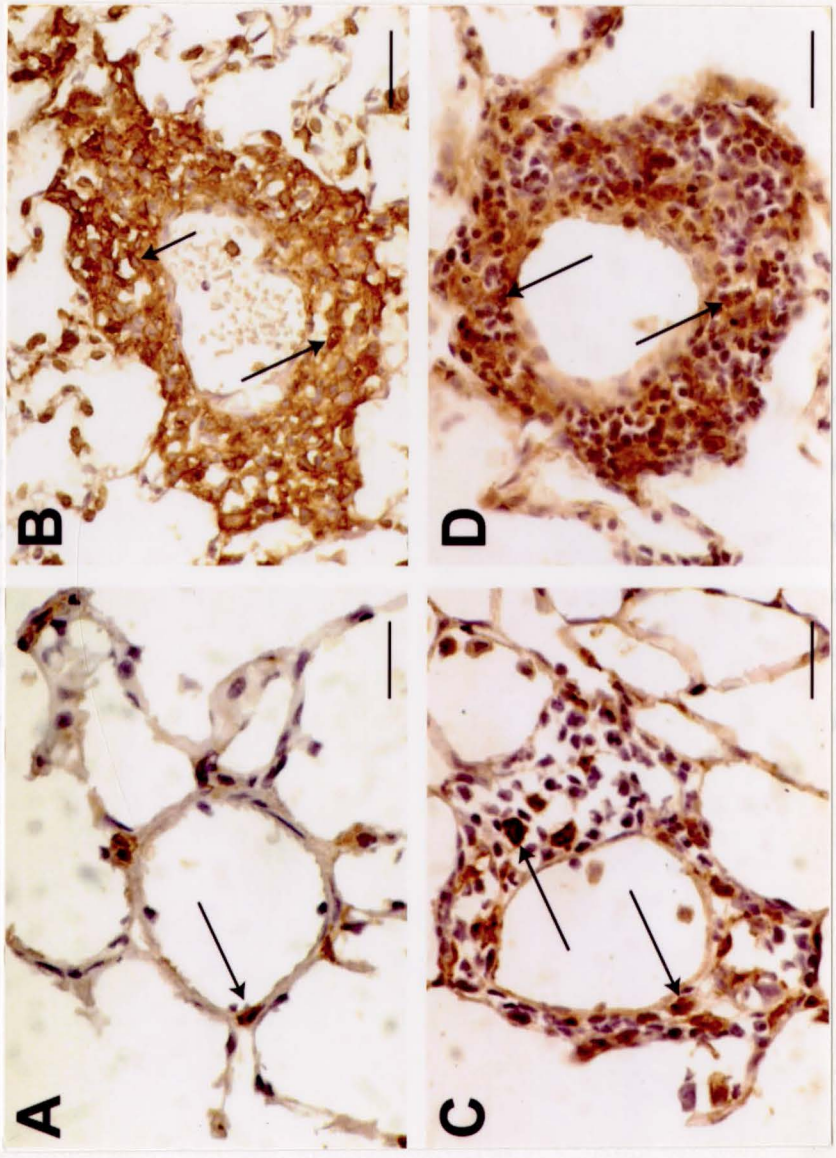
GVHD prior to the development of clinical symptoms.

Table 20. - - Monoclonal mouse anti-rat antibodies, dilutions, target cells and LUT (Look-up Table) lower limit values used for immunohistochemical Image Analysis of lung perivascular mononuclear infiltrates throughout acute GVHD

mAbs	Dilution	Target Cells	LUT Value
MRC OX-6	1:150	MHC class II (Ia)	163
MRC OX-8	1:150	Cytotoxic/Suppressor T cells	182
MRC OX-12	1:110,000	IgG Kappa Chain	N.Q*
MRC OX-19/52	1:150	Pan T cells	190
MRC OX-39	1:150	IL-2r on activated T cells	N.Q*
MRC OX-41	1: 5,000	Alveolar/activated macrophages	190
ED1	1:150	Macrophages/monocytes	179
W3/25	1:150	T helper/macrophages	169
W3/13	1: 2,500	T cells/NK cells	194
MOM/3F12/F2	1:10,000	Granulocytes	N.Q*

*N.Q - No quantifiable antibody labeling occurred within the perivascular space

FIGURE 8. Photomicrographs representative of immunohistochemical labeling within the perivascular adventitial spaces of lung tissues throughout acute GVHD. (A) OX6-labeled mononuclear cell within control tissues (bar indicates 20 μm scale); (B) OX6-labeled mononuclear cells within the advanced stages of day 14 perivascular infiltration (bar indicates 30 μm scale); (C) ED1-labeled mononuclear cells within the early stages of day 14 perivascular infiltration (bar indicates 20 μm scale); (D) OX41-labeled mononuclear cells within the early stages of perivascular infiltration between days 15-21 (bar indicates 20 μm scale).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A
C

FIGURE 9. Photomicrographs representative of immunohistochemical labeling within the perivascular adventitial spaces of lung tissues throughout acute GVHD. (A) OX8-labeled mononuclear cell within the early stages of day 14 perivascular infiltration (bar indicates 30 μm scale); (B) W3/13-labeled mononuclear cells within the perivascular infiltration characteristic of day 14 (bar indicates 20 μm scale); (C) W3/25-labeled mononuclear cells within the perivascular infiltration characteristic of day 14 (bar indicates 20 μm scale); (D) OX19/52-labeled mononuclear cells within the early stages of day 14 perivascular infiltration (bar indicates 20 μm scale).

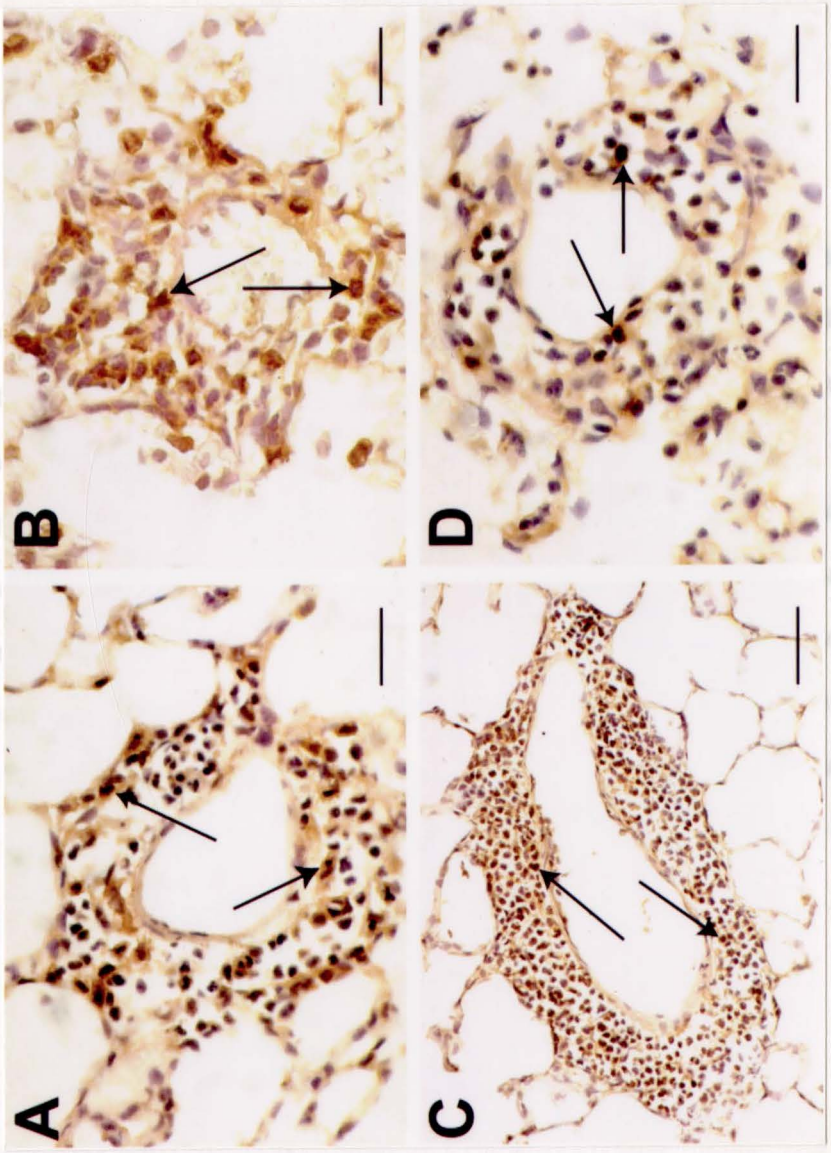

B
D
A
C

FIGURE 10. (A) Perivascular infiltrate volume density of OX-6-labeled mononuclear cells in noninjected (DA×LEW) F_1 controls (day 0), and DA/F_1 GVHD-induced, nonirradiated adult rats 3, 7, 10, 14, and 15-21 days following injection. Data were obtained using quantitative image analysis. Error bars represent means ± SEM. Statistical analyses were conducted using one-way ANOVA and Tukey post-hoc testing. (*) Control volume density measurements were increased over day 3 values because minimal control perivascular spaces contained 1-3 OX6-stained cells. Day 3 values were lower than control volume density measurements because the number of OX6-labeled cells remained at control values, but there was an overall increase in perivascular volume (■)=$P \leq 0.05$ and (■■)=$P \leq 0.01$ as compared to control values. N=4 animals per time point. 480 tissue sites were assessed for quantitation.

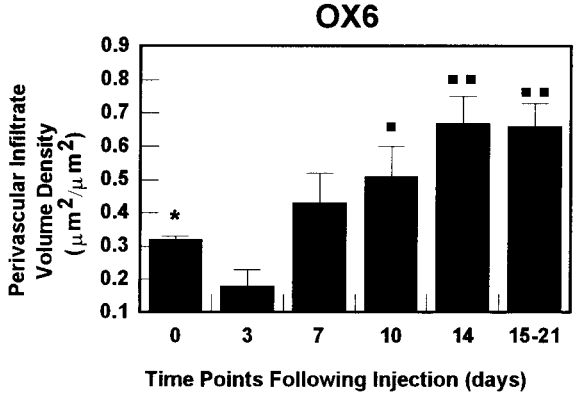

OX6
Perivascular Infiltrate Volume Density ($\mu m^2/\mu m^2$)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
*
0
3
7
10
14
15-21
Time Points Following Injection (days)

Figure 10

FIGURE 11. Perivascular infiltrate volume density of (A) ED1- and (B) OX41-stained mononuclear cells in noninjected (DA×LEW) F_1 controls (day 0), and DA/F_1 GVHD-induced, nonirradiated adult rats 3, 7, 10, 14, and 15-21 days following injection. Data were obtained using quantitative image analysis. Error bars represent means ± SEM. Statistical analyses were conducted using a one-way ANOVA and Tukey post-hoc testing. (A) ED1-labeled cells were not observed within the perivascular spaces of control or day 3 tissues (■■)=$P \leq 0.01$ as compared to day 7 values. (B) OX41-labeled cells were not observed within the perivascular spaces of control, 3, 7, and 10 day tissues N=4 animals per time point. 480 tissue sites were assessed for quantitation.

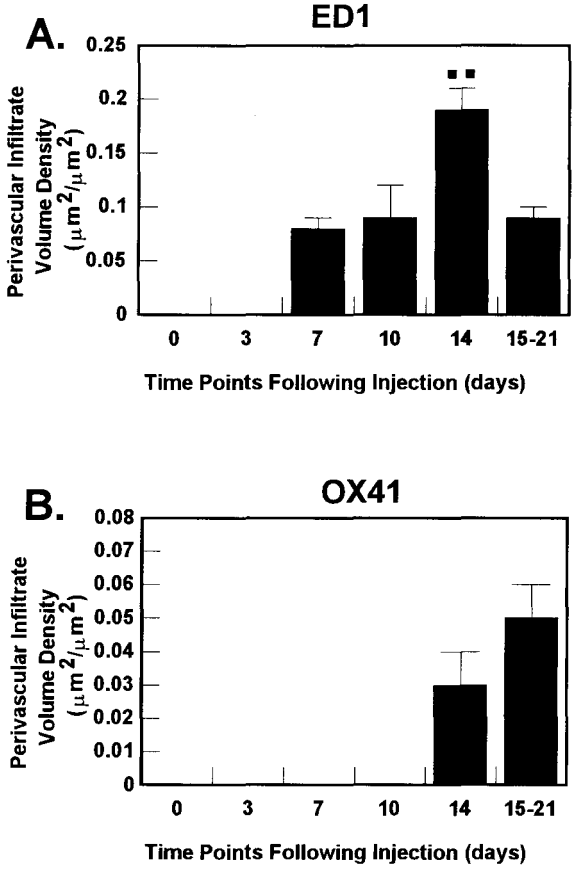

A.
ED1
0.25
0.2
0.15
0.1
0.05
0
Perivascular Infiltrate Volume Density ($\mu m^2/\mu m^2$)
0
3
7
10
14
15-21
Time Points Following Injection (days)
B.
OX41
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Perivascular Infiltrate Volume Density ($\mu m^2/\mu m^2$)
0
3
7
10
14
15-21
Time Points Following Injection (days)

Figure 11

FIGURE 12. Perivascular infiltrate volume density of (A) OX8- and (B) W3/13-stained mononuclear cells in noninjected (DA×LEW) F_1 controls (day 0), and DA/F_1 GVHD-induced, nonirradiated adult rats 3, 7, 10, 14, and 15-21 days following injection. Data were obtained using quantitative image analysis. Error bars represent means ± SEM. Statistical analyses were conducted using a one-way ANOVA and Tukey post-hoc testing. (A) OX8-labeled cells were not observed within the perivascular spaces of control or day 3 tissues. (■)=$P \leq 0.05$ and (■■)=$P \leq 0.01$ as compared to day 7 values. (B) W3/13-labeled cells were not observed within the perivascular spaces of control, 3, 7, and 10 day tissues (■)=$P \leq 0.05$ and (■■)=$P \leq 0.01$ as compared to day 14 values. N=4 animals per time point. 480 tissue sites were assessed for quantitation.

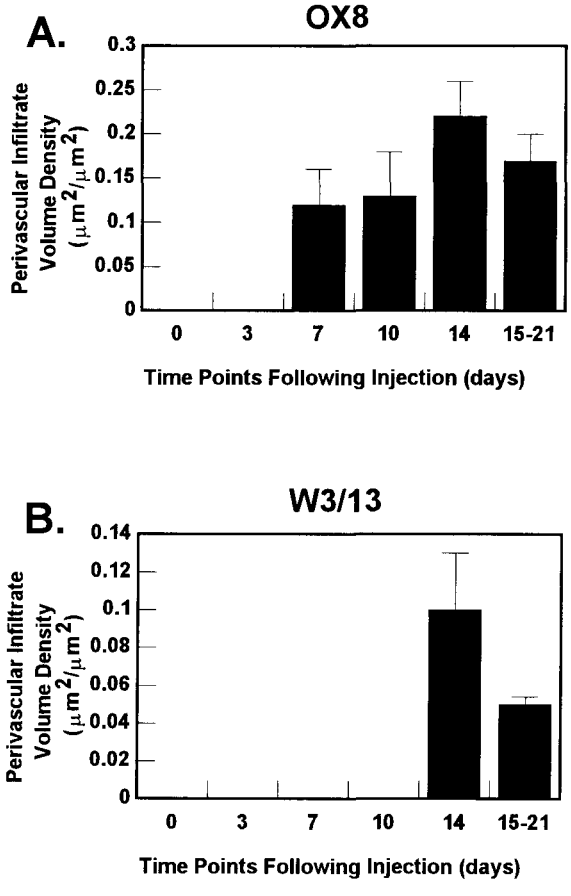
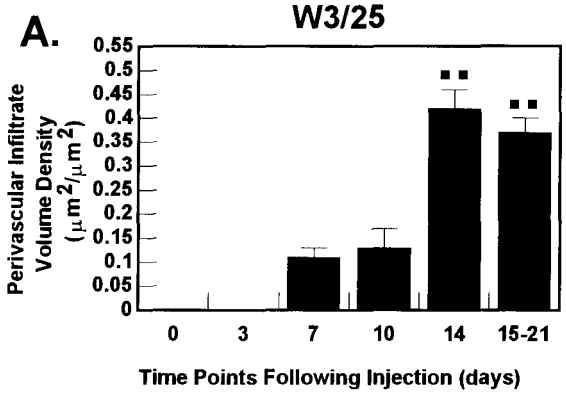

A.
OX8
Perivascular Infiltrate Volume Density ($\mu m^2/\mu m^2$)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
3
7
10
14
15-21
Time Points Following Injection (days)
B.
W3/13
Perivascular Infiltrate Volume Density ($\mu m^2/\mu m^2$)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
3
7
10
14
15-21
Time Points Following Injection (days)

Figure 12

FIGURE 13. Perivascular infiltrate volume density of (A) W3/25- and (B) OX19/52-stained mononuclear cells in noninjected (DA×LEW) F_1 controls (day 0), and DA/F_1 GVHD-induced, nonirradiated adult rats 3, 7, 10, 14, and 15-21 days following injection. Data were obtained using quantitative image analysis. Error bars represent means ± SEM. Statistical analyses were conducted using a one-way ANOVA and Tukey post-hoc testing. (A) W3/25-labeled cells were not observed within the perivascular spaces of control or day 3 tissues (■)=$P \leq 0.05$ and (■■)=$P \leq 0.01$ as compared to day 7 values. (B) OX19/52-labeled cells were not observed within the perivascular spaces of control, 3, 7, and 10 day tissues (■)=$P \leq 0.05$ and (■■)=$P \leq 0.01$ as compared to day 14 values. N=4 animals per time point. 480 tissue sites were assessed for quantitation


A.
W3/25
Perivascular Infiltrate Volume Density ($\mu m^2/\mu m^2$)
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
3
7
10
14
15-21
Time Points Following Injection (days)

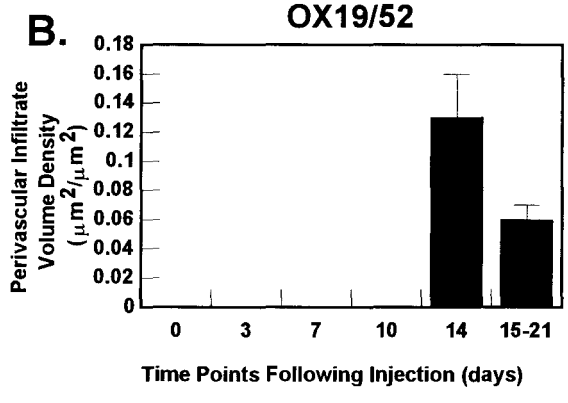

B.
OX19/52
Perivascular Infiltrate Volume Density ($\mu m^2/\mu m^2$)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
3
7
10
14
15-21
Time Points Following Injection (days)

Figure 13

CHAPTER VI

DISCUSSION

The studies described within this dissertation have confirmed the original hypothesis that the lung is an important target organ of acute lethal GVHD. The experimental designs of Chapter's III, IV and V were based on a parental $\rightarrow F_1$ model of acute GVHD that omitted the influence of irradiation, chemotherapy, immunosuppressive drugs or overt infection. Adult (DA × LEW) F_1 hybrid rats were injected with 1×10^6 DA parental lymphoid cells/gram body weight which produced 100% morbidity and mortality by day 21.

The data described in Chapter IV has confirmed the hypothesis that interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis occur as direct result of acute GVHD. Studies were based on the histological analysis of whole lobe lung sections (4 μm) stained with H&E, and the subsequent quantification of variations within alveolar septal width as well as perivascular volume density using light microscopic Image Analysis. Alveolar septal width and perivascular infiltrate volume density values were significantly increased above controls by day 7, and reached 2.4 and 2.6 fold increases respectively by day 21. Between days 15 - 21, the consolidation of perivascular mononuclear cell and alveolar infiltrates marked the onset of interstitial pneumonitis. The bronchiolar mononuclear cell infiltrates that characterize lymphocytic bronchiolitis/bronchitis were observed around terminal and respiratory bronchioles by day 10, fully developed between days 15 - 21, and occurred independently of interstitial pneumonitis. Furthermore, the onset of an acute GVHD-induced interstitial pneumonitis appeared to describe the "idiopathic pneumonitis" of previously undefined origin.

Consequently, Chapter IV produced data that fulfilled several primary objectives of this dissertation (see Chapter I, Introduction p. 2). Time course - The pulmonary histopathology of acute GVHD was observed by day 7 and became progressively more severe until day 21. Anatomical location - The first signs of pathogenesis began in the lung periphery around venules as well as arterioles, and advanced to include increasingly larger vessels as well as bronchioles between days 15 - 21. Histological progression - Alveolar septal thickening and perivascular mononuclear cell infiltration began by day 7, and preceded the onset of lymphocytic bronchiolitis during late-stage GVHD. The degree of pulmonary mononuclear infiltration and edema observed in Chapter IV was corroborated by an increase in lung cell yields as described in Chapter III.

Chapter IV also provided two important insights into the development of acute GVHD-induced histopathology. Histological analysis of the data obtained from Chapter IV revealed that intravascular mononuclear cells displayed an affinity/adherence for the vascular endothelium by day 7. This phenomenon of intravascular adherence occurred prior to perivascular infiltration and was most pronounced on days 7 and 10. In addition, the pulmonary histopathology produced as a direct result of acute GVHD conformed to the grading system applied during the assessment of lung allograft rejection. Consequently, the development of interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis that duplicated the histopathology of lung allograft rejection provided the first evidence to implicate the lung as a target organ of acute GVHD in the adult F_1 hybrid rat.

Further evidence to implicate the lung as a target organ was supplied by the data obtained in Chapter V. This study was based on the immunohistochemical analysis of perivascular mononuclear cell infiltrates that characterize interstitial pneumonitis. The

phenotypic analysis of perivascular infiltrates conducted in Chapter V fulfilled the last stated objective of this dissertation (see Chapter I, Introduction p. 2). Whole left lungs were frozen, serially sectioned (4 μm) and incubated with a wide panel of mouse anti-rat monoclonal antibodies. The density of labeled antibodies within the perivascular space was quantified by light microscopy using Image Analysis software. The data determined that perivascular infiltration occurred in a biphasic pattern of first ED1$^+$, OX8$^+$, and W3/25$^+$ cells, then OX41$^+$, W3/13$^+$, and OX19/52$^+$ populations. OX6 was expressed in control tissues and during all time points throughout acute GVHD. OX12$^+$, OX39$^+$, and MOM/3F12/F2$^+$ cells were not quantifiable within perivascular infiltrates at any time point tested. Overall, volume density fluctuations occurred within 7 out of the 10 antibodies used. These results indicated that acute GVHD-induced pulmonary histopathology was a class II$^+$, T cell and macrophage mediated response.

Chapter V also determined that OX6, OX8, ED1 and W3/25 antibodies labeled the adherent intravascular mononuclear cells observed on day 7 as described in Chapter IV. OX41$^+$-, W3/13$^+$- and OX19/52$^+$ - labeled mononuclear cells did not display an affinity/adherence for the vascular endothelium until day 10, and were not observed within the perivascular space until day 14. The results of Chapter V also showed that OX6$^+$, ED1$^+$, and OX41$^+$ populations have a preferential affinity for the perivascular limiting plate which contains a high concentration of Type IV collagen. The affinity of mononuclear cells containing Type IV collagenase for the limiting plate has suggested a mechanism for dissolution of the limiting plate and onset of interstitial pneumonitis.

Chapters IV and V have also provided indirect evidence to suggest that the intravascular affinity/adherence of mononuclear cells is induced by the presence of activated intravascular donor T cells that release cytokines which upregulate adhesion molecules. These

activated donor cells may be the source of cytokines such as IFN-γ, TNF-α, IL-1 and IL-4 that induce class II antigen expression, upregulate endothelial adhesion molecules and promote perivascular infiltration. The potential upregulation of LFA-1/ICAM-1, ELAM-1, VCAM-1 and VLA adhesion molecules may explain the affinity/adherence of intravascular mononuclear cells and may be a factor in guiding infiltrated cells to the perivascular limiting plate.

The compiled data of Chapter's IV and V correlate well to the cutaneous, intestinal and hepatic immunopathology of acute GVHD. Each of the 3 primary target organs undergoes an increase in class II expression that is accompanied by a mononuclear infiltrate with a high proportion of $CD8^+$ T cells. Skin and intestinal tract are also influenced by an increase in IFN-γ as well as TNF-α, and show an upregulation of ICAM-1, ELAM-1 and VCAM-1. The augmentation of pulmonary class II^+ labeling, the early infiltration of $OX8^+$ T cells, the potential upregulation of cytokine production, and the observed affinity/adherence of intravascular mononuclear cells all appear analogous to the immunopathology that characterizes target organs of acute GVHD. Consequently, comparisons between the immunopathology produced within lungs and other established target organs also appeared to confirm that the lung is a target organ of acute GVHD.

Future studies should be directed toward: (1) identifying the individual cytokines that may be produced by activated intravascular donor T cells, (2) identifying the adhesion molecules produced by those cytokines, (3) defining the synergistic relationship between CMV-induced and GVHD-induced interstitial pneumonitis, and (4) developing a method to detect the onset of acute pulmonary GVHD. In situ hybridization would be useful for identifying individual cytokines and adhesion molecules. While studies combining the administration of anti-ICAM-1 and active CMV may determine if a donor T cell/ICAM-1 complex facilitates the transportation of

CMV into the privileged perivascular space. In addition, a method needs to be developed that can access the lung periphery at multiple tissue sites early after allogeneic BMT. Data from Chapter III has demonstrated that FACS analysis of BAL cell suspensions can not detect the onset or predict the severity of acute GVHD-induced histopathology.

CHAPTER VII

SUMMARY

This dissertation has determined that the lung is an important target organ of acute GVHD. Data has been produced to demonstrate that: (1) FACS analysis of BAL cell suspensions may be able to suggest an ongoing immune process, but in the absence of transbronchial biopsy this method is incapable of determining the onset or progression of acute pulmonary GVHD prior to clinical symptoms occurring from the significant deterioration of normal pulmonary structure, (2) IP and LB occur as a direct result of acute GVHD, (3) IP develops prior to LB and is produced by the coalescence of perivascular and alveolar mononuclear cell infiltrates, (4) acute GVHD-induced IP and LB duplicate the histopathology of lung allograft rejection, and (5) the process of perivascular infiltration is characterized by a biphasic influx of class II^+, T cell and macrophage populations.

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VITA

Diane has been affiliated with Loyola University for over 20 years. In 1977, Diane completed 3 years of Emergency Medical Technician/Paramedic training at Loyola, graduated at the top of her class, and participated in an elite 6-member paramedic team requested to establish new Paramedic/Advanced Life Support programs in other states. In 1979, she became Senior Paramedic/Personnel manager for Town & Country Ambulance Corporation. While at Town & Country, Diane was featured in Loyola World magazine, and appeared in the Chicago Sun Times as well as numerous local newspapers.

While off-duty, Diane was credited with saving the life of a man involved in a car/train collision in Des Plaines. As a result, Chief Charles Gedroic of the Des Plaines Fire Department (DPFD) requested that Diane be the first female paramedic to join a full-time fire department in the State of Illinois. As Senior Paramedic/Paramedic Coordinator of DPFD, Diane carried out the department-wide update and re-organization of trauma equipment used on advanced life support vehicles. She led a full team of paramedics on the ambulance and an engine company of firefighter emergency medical technicians (FFEMT's) during emergency calls. Diane also directed the certification and re-certification of paramedics and FFEMT's within the Northwest Community Hospital and Loyola, Foster G. McGaw emergency medical systems.

While at DPFD, Diane was selected as a member of the search and rescue team employed in the Rosemont Stadium collapse disaster and was in the first responding

emergency unit to the United Airlines Flight 191 air disaster. Diane also designed, developed and produced the "Respiratory Roll-up", a compartmentalized, easy-access carrying case for the management of emergency respiratory equipment by advanced life support personnel that is used nationwide. Throughout her paramedic career Diane received numerous commendations for outstanding performance from local fire departments, public officials, and patients.

In 1980, Diane began her undergraduate education at the Lake Shore Campus while working at the medical center as a surgical technician for the Department of Anesthesiology. Diane matriculated from Loyola with bachelors degrees in English and Fine Arts (Medical Illustration/Advertising). While completing her undergraduate education, Diane received a Carnegie Mellon award for achievement in black & white photography, and a monetary award from Loyola for outstanding work displayed in the 1986 Spring Senior Art Exhibit.

Diane was admitted to the graduate program in the Department of Cell Biology, Neurobiology and Anatomy in August 1986. She was the recipient of the University Basic Science Fellowship Award from 1986-1990. She taught in the medical anatomy, histology and neuroscience courses, and participated as Graduate Student Representative to the Faculty as well as Graduate Student Representative for Graduate Admissions from 1988-1990. Since 1993, while completing her research, Diane has worked 32 hours a week for Loyola's Division of Information Technologies as Computing Center Coordinator for the Department of Client Services.

Diane's dissertation work was conducted in the laboratory of Dr. John Clancy, Jr., Chairman of the Department of Cell Biology, Neurobiology and Anatomy. In addition to

developing new photographic and histologic techniques, her first manuscript is considered to be a landmark publication in the field of Graft-vs.-Host Disease.

DISSERTATION APPROVAL SHEET

The dissertation submitted by Diane L. Workman has been read and approved by the following committee:

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Ph.D.

April 5, 1995
Date


Director's Signature